SPECIFICATION

METHOD FOR PRODUCING TRIPEPTIDES AND/OR PEPTIDES LONGER THAN TRIPEPTIDES

5

TECHNICAL FIELD

The present invention relates to a method for producing peptides that can produce peptides easily, inexpensively and at high yield without going through a complex synthetic method. More particularly, the present invention

10 relates to a method for producing peptides that are equal to or longer than tripeptides by using an enzyme that catalyzes a peptide-forming reaction from a carboxy component and an amine component.

BACKGROUND ART

15 Peptides are used in the fields of pharmaceuticals, foods and various other fields. For example, since L-alanyl-L-glutamine has higher stability and water-solubility than L-glutamine, it is widely used as a component of fluid infusion and serum-free media.

Chemical synthesis methods, which have been known as methods for

20 producing peptides, are not always easy. Known examples of such methods include a method that uses N-benzyloxycarbonylalanine (hereinafter, "Z-alanine") and protected L-glutamine (see Bull. Chem. Soc. Jpn., 34, 739 (1961), Bull. Chem. Soc. Jpn., 35, 1966 (1962)), a method that uses Z-alanine and protected L-glutamic acid-γ-methyl ester (see Bull. Chem. Soc. Jpn., 37,

25 200 (1964)), a method that uses Z-alanine ester and unprotected glutamic acid

(see Japanese Patent Application Laid-open Publication No. H1-96194), a method that involves synthesis of an N-(2-substituted)-propionyl glutamine derivative as an intermediate from a 2-substituted-propionyl halide as a raw material (see Patent Application Laid-open Publication No. H6-234715).

5   However, since all of these methods require the introduction and elimination of protecting groups or the use of an optically active intermediate, they are not considered to be adequately satisfactory in terms of their industrial advantages.

On the other hand, widely known examples of typical peptide

10   production methods using enzymes consist of a condensation reaction that uses an N-protected, C-unprotected carboxy component and an N-unprotected, C-protected amine component (hereinafter, "Reaction 1"), and a substitution reaction that uses an N-protected, C-protected carboxy component and an N-unprotected, C-protected amine component (hereinafter, "Reaction 2"). An

15   example of Reaction 1 is a method for producing Z-aspartylphenylalanine methyl ester from Z-aspartic acid and phenylalanine methyl ester (see Japanese Patent Application Laid-open Publication No. S53-92729), while an example of Reaction 2 is a method for producing acetylphenylalanylleucine amide from acetylphenylalanine ethyl ester and leucine amide (see

20   Biochemical J., 163, 531 (1977)). There have been reported very few research examples of methods that use an N-unprotected, C-protected carboxy component. An example of a substitution reaction that uses an N-unprotected, C-protected carboxy component and an N-unprotected, C-protected amine component (hereinafter, "Reaction 3") is described in

25   International Patent Publication WO 90/01555. For example, a method for

producing arginylleucine amide from arginine ethyl ester and leucine amide may be mentioned of. Examples of substitution reactions that use an N-unprotected, C-protected carboxy component and an N-unprotected, C-unprotected amine component (hereinafter, "Reaction 4") are described in

5 European Patent Publications EP 278787A1 and EP 359399B1. For example, a method for producing tyrosylalanine from tyrosine ethyl ester and alanine may be mentioned of.

DISCLOSURE OF THE INVENTION

10 The most inexpensive production method among the aforementioned methods of Reactions 1 to 4 naturally falls within the class of Reaction 4, which involves the fewest protecting groups.

However, the example of Reaction 4 of the prior art (European Patent Publication EP 278787A1) had the following major problems: (1) extremely

15 slow rate of peptide production, (2) low peptide production yield, (3) the peptides that can be produced are limited to those that contain amino acids with comparatively high hydrophobicity, (4) the amount of enzyme added is extremely large, and (5) comparatively expensive carboxypeptidase preparations derived from molds, yeasts or plants are required. In the

20 Reaction 4, there is no method known whatsoever that uses an enzyme derived from bacteria or yeasts other than the genus Saccharomyces, and there is no known method for producing alanylglutamine and other peptides that are highly hydrophilic. In consideration of this background, there is a need to develop an industrially inexpensive method for producing these

25 peptides.

In addition, the method for producing peptides of the Reaction 4 that uses an enzyme is limited to the formation of dipeptides, and there has been a need to develop a method for simply and easily producing peptides that are equal to or longer than tripeptides so that they can be adequately used

5   industrially.

Under the circumstances, it is an object of the present invention to provide a method that can produce peptides that are equal to or longer than tripeptides to easily, inexpensively and at high yield without going through a complex synthesis method.

10   As a result of conducting extensive research in consideration of the aforementioned object, the inventors of the present invention have discovered an enzyme that efficiently forms peptides that are equal to or longer than tripeptides from newly discovered bacteria belonging to the genus *Empedobacter* and bacteria belonging to the genus *Sphingobacterium*, and

15   have completed the present invention.

Namely, the present invention is as described below.

[1]   A method for producing a peptide having three or more amino acid residues including the step of forming the peptide having three or more amino acid residues with an enzyme or enzyme-containing substance, wherein the

20   enzyme or enzyme-containing substrate has an ability to use as substrates an amine component having two or more amino acid residues and a carboxy component, to form a peptide having one more peptide bond than the amine component.

[2]   The method for producing a peptide according to [1], wherein the

25   enzyme or enzyme-containing substance includes one type or two or more

types selected from the group consisting of a culture of a microbe, microbial cells separated from the culture, and a treated microbial cell product of the microbe which have the ability to use as substrates an amine component having two or more amino acid residues and a carboxy component, to form a

5   peptide having one more peptide bond than the amine component.

[3]   The method for producing a peptide according to [1] or [2], wherein the enzyme or enzyme-containing substance is able to use as the carboxy component both an amino acid ester and an amino acid amide .

[4]   The method for producing a peptide according to any one of [1] to [3],

10   wherein the enzyme or enzyme-containing substance is able to use as the amine component any of (i) an unprotected peptide having two or more amino acid residues, (ii) a C-protected peptide having two or more amino acid residues, and (iii) a peptide having two or more amino acid residues whose C-terminal molecule is an amine instead of an amino acid.

15   [5]   The method for producing a peptide according to [1], wherein the enzyme is a protein (A) or (B):

(A) a protein having an amino acid sequence consisting of amino acid residues numbers 23-616 of an amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing,

20   (B) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues 23 to 616 of the amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing, and having activity to use as substrates an amine component having two or more amino

25   acid residues and a carboxy component, to form a peptide having one more

peptide bond than the amine component.

[6]   The method for producing a peptide according to [1], wherein the enzyme is a protein (C) or (D):

(C) a protein having an amino acid sequence consisting of amino acid residues numbers 21 to 619 of an amino acid sequence described in SEQ ID NO: 12 of the Sequence Listing,

(D) a protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues 21 to 619 of the amino acid sequence described in SEQ ID NO: 12 of the Sequence Listing, and having activity to use as substrates an amine component having two or more amino acid residues and a carboxy component, to form a peptide having one more peptide bond than the amine component.

[7]   The method for producing a peptide according to [1], wherein the enzyme is a protein (E) or (F):

(E) a protein having an amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing,

(F) a protein containing a mature protein region, the protein having an amino acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing, and having activity to use as substrates an amine component having two or more amino acid residues and a carboxy component, to form a peptide having one more peptide bond than the amine component.

[8]   The method for producing a peptide according to [1], wherein the

enzyme is a protein (G) or (H):

(G) a protein having an amino acid sequence described in SEQ ID NO: 12 of the Sequence Listing,

(H) a protein containing a mature protein region, the protein having an amino
5   acid sequence including substitution, deletion, insertion, addition, and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 12 of the Sequence Listing but contains therein substitution, deletion, insertion, addition and/or inversion of one or a plurality of amino acids and having activity to use as substrates an amine component
10   having two or more amino acid residues and a carboxy component, to form a peptide having one more peptide bond than the amine component.

[9]   The method for producing a peptide according to [2], wherein the microbe is a microbe belonging to the genus *Empedobacter* or belonging to the genus *Sphingobacterium*.

15   [10]   The method for producing a peptide according to [2], wherein the microbe is a microbe that has been transformed so as to be able to express a protein encoded by a DNA (a) or (b):

(a) a DNA that having a base sequence consisting of bases numbers 127 to 1908 of a base sequence described in SEQ ID NO: 5 of the Sequence Listing,
20   (b) a DNA that hybridizes with a DNA having a base sequence complementary to the base sequence consisting of bases numbers 127 to 1908 of the base sequence described in SEQ ID NO: 5 of the Sequence Listing under stringent conditions, and encodes a protein that has peptide-forming activity.

25   [11]   The method for producing a peptide according to [2], wherein the

microbe is a microbe that has been transformed so as to be able to express a protein encoded by a DNA (c) or (d):

(c) a DNA that consists of bases numbers 121 to 1917 of a base sequence described in SEQ ID NO: 11 of the Sequence Listing,

5   (d) a DNA that hybridizes with a DNA having a base sequence complementary to the base sequence consisting of bases numbers 121 to 1917 of the base sequence described in SEQ ID NO: 11 of the Sequence Listing under stringent conditions, and encodes a protein that has peptide-forming activity.

10   [12]   The method for producing a peptide according to [2], wherein the microbe is a microbe that has been transformed so as to be able to express protein encoded by a DNA (e) or (f):

(e) a DNA that consists of bases numbers 61 to 1908 of a base sequence described in SEQ ID NO: 5 of the Sequence Listing,

15   (f) a DNA that hybridizes with a DNA having a base sequence complementary to the base sequence consisting of bases numbers 61 to 1908 of the base sequence described in SEQ ID NO: 5 of the Sequence Listing under stringent conditions, and encodes a protein that has peptide-forming activity.

20   [13]   The method for producing a peptide according to [2], wherein the microbe is a microbe that has been transformed so as to be able to express a protein encoded by a DNA (g) or (h):

(g) a DNA that consists of bases numbers 61 to 1917 of a base sequence described in SEQ ID NO: 11 of the Sequence Listing,

25   (h) a DNA that hybridizes with a DNA that consisting of a base sequence

complementary to the base sequence consisting of bases numbers 61 to 1917

of the base sequence described in SEQ ID NO: 11 of the Sequence Listing

under stringent conditions, and encodes a protein that contains a mature

protein region that has peptide-forming activity.

5   [14]   The method for producing a peptide according to any one of [1] to [13],

wherein the carboxy component comprises one type or two or more types

selected from the group consisting of an L-alanine ester, a glycine ester, an

L-threonine ester, an L-tyrosine ester and a D-alanine ester.

10   BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the optimum pH of an enzyme of *Empedobacter*;

Fig. 2 shows the optimum temperature of an enzyme of

Empedobacter;

Fig. 3 shows the time course of L-alanyl-L-glutamine production from

15   L-alanine methyl ester and L-glutamine; and

Fig. 4 shows amounts of an enzyme existing in a cytoplasm fraction

(Cy) and a periplasm fraction (Pe).

BEST MODE FOR CARRYING OUT THE INVENTION

20   The method for producing a peptide that is equal to or longer than a

tripeptide according to the present invention is explained in detail in the order

of:

1. Method for Producing Peptides that are Equal to or Longer than

Tripeptides, and

25   2. Enzyme Used in the Present Invention.

In the present specification, the carboxy component refers to the component that provides a carbonyl site (CO) in the peptide bond (-CONH-), while the amine component refers to the component that provides the amino site (NH) in the peptide bond. In addition, in the present specification, the

5   term "peptide" when used alone refers to a polymer having at least one peptide bond unless otherwise indicated specifically. Further, the term "dipeptide" herein refers to a peptide having one peptide bond. Moreover, the term "a peptide that is equal to or longer than a tripeptide" herein refers to a peptide having two or more peptide bonds.

10

1. Method for Producing Peptides that are Equal to or Longer than Tripeptides

In the method for producing peptides that are equal to or longer than tripeptides according to the present invention (hereinafter also referred to "peptide production method of the present invention"), a carboxy component

15   and an amine component are allowed to react in the presence of a predetermined enzyme. That is to say, the method for producing peptides according to the present invention employs an enzyme or an enzyme-containing substance that has the ability to form a peptide having one more peptide bond than the amine component, from the amine component

20   equal to or longer than a dipeptide and the carboxy component. That enzyme or enzyme-containing substance acts on the carboxy component and the amine component, to produce the peptide that is equal to or longer than a tripeptide.

The action of the enzyme or enzyme-containing substance on the

25   carboxy component and on the amine component may be accomplished

merely by mixing the enzyme or enzyme-containing substance, the carboxy component and the amine component. More specifically, a method by adding an enzyme or enzyme-containing substance to a solution containing the carboxy component and the amine component and allowing the resultant to

5 react can be used. Alternatively, in the case of using a microbe that produces the enzyme, a method by adding the carboxy component and amine component to a microbial culture broth after the enzyme was produced and accumulated in the microbial cells or in the culture broth can be used. If necessary, the produced peptide that is equal to or longer than a tripeptide can

10 then be recovered and purified by conventional methods.

The term "enzyme-containing substance" means any substance so far as it contains the enzyme, and examples of specific forms thereof include a microbial culture broth containing the enzyme, microbial cells isolated from the culture, and a product obtained by treating microbial cells (hereinafter, "treated

15 microbial cell product"). A microbial culture broth refers to what is obtained by culturing a microbe, and more specifically, to a mixture of microbial cells, medium used for culturing the microbe and substances produced by the cultured microbe, and so forth. In addition, the microbial cells can be used in the form of washed microbial cells. In addition, the treated microbial cell

20 product includes disrupted, lysed or freeze-dried microbial cells, and the like, and also includes a crude enzyme recovered from treated microbial cells, and a purified enzyme obtained by purification of the crude enzyme, and so forth. A partially purified enzyme obtained by various purification methods may be used as the purified enzyme, or immobilized enzymes may be used that have

25 been immobilized by a covalent bonding method, an adsorption method or an

entrapment method. In addition, since some microbes are partially lysed during culturing depending on the microbes used, in such cases, the culture supernatant may also be used as the enzyme-containing substance.

In addition, wild strains may be used for the microbes that contain the enzyme, or recombinant strains that express the enzyme obtained by genetic engineering may also be used. The microbes are not limited to intact microbial cells, but rather acetone-treated microbial cells, freeze-dried microbial cells or other treated microbial cells may also be used. Immobilized microbial cells and an immobilized treated microbial cell product obtained by immobilization by covalent bonding, adsorption, entrapment or other methods, may also be used.

Use of a wild strain producing a peptide-forming enzyme having activity to form a peptide, that is equal to or longer than a tripeptide, is rather preferable since the trouble of construction of a recombinant strain by genetic engineering and so forth is eliminated and a peptide is produced more easily. On the other hand, a recombinant strain obtained by genetic engineering that has been transformed to express a peptide-forming enzyme having activity to form a peptide that is equal to or longer than a tripeptide can produce a larger amount of a peptide. So, synthesis of peptides that are equal to or longer than tripeptides can be carried out more rapidly in large amounts. Peptides that are equal to or longer than tripeptides can be produced by mixing amino acid esters and amine component with wild or recombinant microbial culture broth and/or wild or recombinant microbial cells which is cultured in the medium.

In addition, in the case of microbial culture broth, cultured microbial

cells, washed microbial cells or a treated microbial cell product that has been obtained by disrupting or lysing microbial cells, there are many cases in which an existing enzyme does not participate in peptide formation but decomposes the formed peptides.   In this situation, it may be rather preferable in some

5   cases to add a metal protease inhibitor such as ethylenediaminetetraacetic acid (EDTA).   The addition amount is within the range of 0.1 millimolar (mM) to 300 mM, and preferably 1 mM to 100 mM.

The amount of enzyme or enzyme-containing substance used may be enough if its amount is effective for peptide production (effective amount).

10   This effective amount can be easily determined through simple and preliminary experimentation by a person with ordinary skill in the art.   In the case of using the enzyme, for example, the effective amount is about 0.01 to about 100 units (U), while in the case of using washed microbial cells, the effective amount is about 1 to about 500 grams per liter (g/L).

15   Any carboxy component may be used so far as it can form a peptide by condensation with the amine component that is the other substrate. Examples of the carboxy component include L-amino acid esters, D-amino acid esters, L-amino acid amides and D-amino acid amides.   In addition, examples of the amino acid esters or amino acid amides include amino acid

20   esters or amino acid amides corresponding to not only naturally-occurring amino acids, but also corresponding to non-naturally-occurring amino acids or their derivatives.   In addition, examples of the amino acid esters or amino acid amides include not only α-amino acid esters or α-amino acid amides but also β-, γ- and ω- *etc*. amino acid esters or β-, γ- and ω- *etc*. amino acid amides,

25   which have different binding sites of amino groups.   Typical examples of the

amino acid ester include methyl esters, ethyl esters, n-propyl esters, iso-propyl esters, n-butyl esters, iso-butyl esters, and tert-butyl esters, *etc.* of amino acids.

Any peptide may be used for the amine component so far as it can form a peptide by condensation with the carboxy component that is the other substrate. The minimum unit of the amine component is a dipeptide, while there is no particular upper limit therefor. In addition, there is also no particular restriction on the amino acid sequence of the peptide serving as the amine component. In addition, the peptide used as the amine component may include the one whose side chain was modified, or one that contains amines instead of the amino acids. More specifically, examples of the amine component include C-unprotected peptides being equal to or longer than dipeptides, C-protected peptides being equal to or longer than dipeptides, and peptides being equal to or longer than dipeptides, in which the C-terminal molecule is an amine instead of the amino acid. For example, structure of a tripeptide that is equal to or longer than a dipeptide in which the C-terminal molecule is an amine instead of an amino acid is described as N-terminal amino acid - amino acid - amine.

Although the concentrations of the carboxy component and amine component serving as starting materials are 1 mM to 10 molars (M), and preferably 0.05 M to 2 M, respectively, there are cases in which it is preferable to add the amine component in an amount equimolar or excess molar with respect to the carboxy component. In addition, when high concentrations of substrates inhibit the reaction, step-wise addition without inhibition can be used during the reaction.

The reaction temperature that allows formation of a peptide is 0 to 60°C, and preferably 5 to 40°C. The reaction pH that allows formation of a peptide is 6.5 to 10.5, and preferably 7.0 to 10.0.

To produce a peptide being equal to or longer than a tripeptide having a desired amino acid sequence, it is sufficient that amino acid esters serving as the carboxy components be selected step-wisely to make the desired amino acid sequence, with which the amino acid sequence may be successively extended. For example, to produce a tripeptide, L-Ala-L-His-L-Ala, the tripeptide may be synthesized by using alanine methyl ester as the carboxy component and by using a dipeptide, L-His-L-Ala, as the amine component. After formation of the tripeptide, a peptide, Gly-L-Ala-L-His-L-Ala, can be obtained by adding glycine methyl ester as the carboxy component with the above tripeptide as the amine component.

2. Enzyme Used in the Present Invention

The aforementioned peptide production method of the present invention employs an enzyme having the ability to form a peptide having one more peptide bond than the amine component from the carboxy component and the peptide being equal to or longer than a dipeptide as the amine component. In the peptide production method of the present invention, there is no particular restriction on the origin or the obtaining method and so forth of the enzyme so far as the enzyme has this activity. Hereinafter, microbes having the enzymes that can be used in the present invention, cultivation of the microbes, purification of the enzymes, and application of genetic engineering techniques will be explained.

(2-1) Microbes having Enzyme that can be Used in the Production Method of the Present Invention

Examples of microbes that form the enzyme of the present invention include bacteria belonging to the genus *Empedobacter* and bacteria belonging

5   to the genus *Sphingobacterium*, and more specifically include *Empedobacter brevis* ATCC 14234 strain (FERM P-18545 strain, FERM BP-8113 strain) and *Sphingobacterium* sp. strain FERM BP-8124. *Empedobacter brevis* ATCC 14234 strain (FERM P-18545 strain, FERM BP-8113 strain) and *Sphingobacterium* sp. FERM BP-8124 strain are microbes that the inventors of

10   the present invention isolated through the screening of microbes that produce a peptide from a carboxy component and an amine component in high yield.

Empedobacter brevis ATCC 14234 strain (FERM P-18545 strain, FERM BP-8113 strain) was deposited at the International Patent Organism Depositary of the independent administrative corporation, National Institute of

15   Advanced Industrial Science and Technology (Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan) on October 1, 2001 and assigned the deposit number of FERM P-18545. Control of this organism was subsequently transferred to deposition under the provisions of the Budapest Treaty at the International Patent Organism Depositary of the independent

20   administrative corporation, National Institute of Advanced Industrial Science and Technology on July 8, 2002 and was assigned the deposit number of FERM BP-8113 (indication of microbe: *Empedobacter brevis* AJ 13933 strain).

Sphingobacterium sp. AJ 110003 strain was deposited at the International Patent Organism Depositary of the independent administrative

25   corporation, National Institute of Advanced Industrial Science and Technology

(Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan) on July 22, 2002, and was assigned the deposit number of FERM BP-8124. In addition, the strain AJ 110003 (FERM BP-8124) was identified to be the aforementioned *Sphingomonas* sp. by the identification experiment described

5   hereinbelow. The strain FERM BP-8124 is a Gram-negative rod (0.7 to 0.8 × 1.5 to 2.0 μm) that forms no spore and is not motile. Its colonies are, round with a completely smooth border, contain low protrusions, have a glossy and light yellow color. The strain FERM BP-8124 grows at 30°C and is catalase positive, oxidase positive and the OF test (glucose) negative. Judging from

10 these results, this strain was identified as a bacterium belonging to the genus *Sphingobacterium*. Moreover, from the properties that nitrate reduction is negative, indole production is negative, acid production from glucose is negative, arginine dihydrolase is negative, urease is positive, esculin hydrolysis is positive, gelatin hydrolysis is negative, β-galactosidase is positive,

15 glucose assimilation is positive, L-arabinose assimilation is negative, D-mannose assimilation is positive, D-mannitol assimilation is negative, N-acetyl-D-glucosamine assimilation is positive, maltose assimilation is positive, potassium gluconate assimilation is negative, n-capric acid assimilation is negative, adipic acid assimilation is negative, dl-malic acid

20 assimilation is negative, sodium citrate assimilation is negative, phenyl acetate assimilation is negative and cytochrome oxidase is positive, it was determined to have properties that are similar to those of *Sphingobacterium multivorum* or *Sphingobacterium spiritivorum*. Moreover, although results of analyses on the homology of the base sequence of the 16S rRNA gene indicate the highest

25 degree of homology with *Sphingobacterium multivorum* (98.8%), there was no

strain with which the bacterial strain matched completely. Accordingly, this bacterial strain was identified as *Sphingobacterium* sp.

(2-2) Cultivation of Microbe

To obtain cultured cells of microbes having the enzyme used in the present invention, it suffices that the microbes is cultured and grown in a suitable medium. There is no particular restriction on the medium used for this purpose so far as it is allows the microbes to grow. This medium may be an ordinary medium containing ordinary carbon sources, nitrogen sources, phosphorus sources, sulfur sources, inorganic ions, and organic nutrient sources as necessary.

For example, any carbon source may be used so far as the microbes can utilize it. Specific examples of the carbon source that can be used include sugars such as glucose, fructose, maltose and amylose, alcohols such as sorbitol, ethanol and glycerol, organic acids such as fumaric acid, citric acid, acetic acid and propionic acid and their salts,hydrocarbons such as paraffin as well as mixtures thereof.

Examples of nitrogen sources that can be used include ammonium salts of inorganic salts such as ammonium sulfate and ammonium chloride, ammonium salts of organic acids such as ammonium fumarate and ammonium citrate, nitrates such as sodium nitrate and potassium nitrate, organic nitrogen compounds such as peptone, yeast extract, meat extract and corn steep liquor as well as their mixtures.

In addition, ordinary nutrient sources used in media, such as inorganic salts, trace metal salts and vitamins, can also be suitably mixed and used.

There is no particular restriction on culturing conditions, and cultivation

can be carried out, for example, for about 12 to about 48 hours while properly controlling the pH and temperature with a pH range of 5 to 8 and a temperature range of 15 to 40°C, respectively, under aerobic conditions.

(2-3) Enzyme Purification

5   As previously mentioned, the peptide-forming enzyme used in the present invention can be purified from bacteria belonging to, for example, the genus *Empedobacter*.   A method for isolating and purifying a peptide-forming enzyme from *Empedobacter brevis* is explained as an example of purification of the enzyme.

10   First, a microbial cell extract is prepared from microbial cells of *Empedobacter brevis*, for example, the strain FERM BP-8113 (Depositary institution: the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary, Address of depositary institution: Chuo Dai-6, 1-1 Higashi

15 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date: July 8, 2002) by disrupting the cells using a physical method such as ultrasonic disruption or an enzymatic method using a cell wall-dissolving enzyme and removing the insoluble fraction by centrifugal separation and so forth.   The peptide-producing enzyme can then be purified by fractionating the cell extract

20 containing the enzyme obtained in the above manner by combining ordinary protein purification methods such as anion exchange chromatography, cation exchange chromatography or gel filtration chromatography.

An example of a carrier for use in anion exchange chromatography is Q-Sepharose HP (manufactured by Amersham).   The enzyme is recovered in

25 the non-adsorbed fraction under conditions of pH 8.5 when the cell extract

containing the enzyme is applied to a column packed with the carrier.

An example of a carrier for use in cation exchange chromatography is MonoS HR (manufactured by Amersham). After adsorbing the enzyme onto the column by applying the cell extract containing the enzyme to a column

5   packed with the carrier and then washing the column, the enzyme is eluted with a buffer solution containing a high salt concentration. At that time, the both methods that the salt concentration is step-wisely increased or is gradiently increased are available. For example, in the case of using MonoS HR, the enzyme adsorbed onto the column is eluted at an NaCl concentration

10   of about 0.2 to about 0.5 M.

The enzyme purified in the manner described above can then be further purified with homogeneity by gel filtration chromatography and so forth. An example of the carrier for use in gel filtration chromatography is Sephadex 200pg (manufactured by Amersham).

15   In the aforementioned purification procedure, the fraction containing the enzyme can be verified by assaying the peptide-forming activity of each fraction according to the method indicated in the examples to be described later. The internal amino acid sequence of the enzyme purified in the manner described above is shown in SEQ ID NO: 1 and SEQ ID NO: 2 of the

20   Sequence Listing.

In addition, a preferable mode of the enzyme of the present invention is an enzyme having the property to be able to use both an amino acid ester and an amino acid amide as a substrate for the carboxy component. The words "both an amino acid ester and an amino acid amide can be used as a

25   substrate" means that at least one or more types of amino acid ester and at

least one or more types of amino acid amide can be used as a substrate. In addition, another preferable mode of the enzyme of the present invention is an enzyme having the property to be able to use all of a C-unprotected peptide being equal to or longer than a dipeptide, a C-protected peptide being equal to

5   or longer than a dipeptide, and a peptide being equal to or longer than a dipeptide in which the C-terminal molecule is an amine instead of an amino acid as a substrate for the amine component. The words "the ability to use all of a C-unprotected peptide being equal to or longer than a dipeptide, a C-protected peptide being equal to or longer than a dipeptide, and a peptide

10   being equal to or longer than a dipeptide in which the C-terminal molecule is an amine instead of an amino acid" mean that at least one or more types of a C-unprotected peptide being equal to or longer than a dipeptide, at least one or more types of a C-protected peptide being equal to or longer than a dipeptide, and at least one or more types of a peptide being equal to or longer than a

15   dipeptide in which the C-terminal molecule is an amine instead of an amino acid can be used as a substrate. The enzyme of the present invention has a wide range of substrate specificity with respect to the carboxy component or the amino component. The properties are preferable in that a wide range of raw materials can be selected, which in turn is favorable in terms of cost and

20   production equipment in the case of industrial production.

    (2-4) Isolation of DNA, Construction of Transformant and Purification of Peptide-forming Enzyme

      (2-4-1) Isolation of DNA

      The inventors of the present invention first succeeded in isolating one

25   type of DNA encoding a peptide-forming enzyme that can be used in the

peptide production method of the present invention from *Empedobacter brevis*

strain FERM BP-8113 (Depositary institution: the independent administrative

corporation, National Institute of Advanced Industrial Science and Technology,

International Patent Organism Depositary, Address of depositary institution:

5   Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan,

International deposit transfer date: July 8, 2002).   A DNA having a base

sequence consisting of bases numbers 61 to 1908 of the base sequence

described in SEQ ID NO: 5, which is a DNA of the present invention, was

isolated from *Empedobacter brevis* strain FERM BP-8113 (Depositary

10   institution: the independent administrative corporation, National Institute of

Advanced Industrial Science and Technology, International Patent Organism

Depositary, Address of depositary institution: Chuo Dai-6, 1-1 Higashi

1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date:

July 8, 2002).   The DNA having the base sequence consisting of bases

15   numbers 61 to 1908 is a code sequence (CDS) portion.   In the base sequence

consisting of bases numbers 61 to 1908 is contained a signal sequence region

and a mature protein region.   The signal sequence region consists of bases

numbers 61 to 126, while the mature protein region consists of bases numbers

127 to 1908.   That is, the present invention provides both a gene for a peptide

20   enzyme protein that contains a signal sequence, and a gene for a peptide

enzyme protein in the form of a mature protein.   The signal sequence

contained in the sequence described in SEQ ID NO: 5 is a kind of leader

sequence. The main function of a leader peptide encoded by the leader

sequence is presumed to be excretion from inside the cell membrane to

25   outside the cell membrane.   The protein encoded by bases numbers 127 to

1908, namely the site excluding the leader peptide, is a mature protein and exhibits a high activity of peptide-forming.

The DNA consisting of the base sequence that consists of bases numbers 61 to 1917 described in SEQ ID NO: 11, which is also a DNA of the present invention, was isolated from *Sphingobacterium* sp. strain FERM BP-8124 (Depositary institution: the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary, Address of depositary institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit date: July 22, 2002).   The DNA consisting of the base sequence that consists of bases numbers 61 to 1917 is a code sequence (CDS) portion.   In the base sequence consisting of bases numbers 61 to 1917, a signal sequence region and a mature protein region are contained.   The signal sequence region consists of bases numbers 61 to 120, while the mature protein region consists of bases numbers 121 to 1917.   That is, the present invention provides both a gene for a peptide enzyme protein that contains a signal sequence, and a gene for a peptide enzyme protein in the form of a mature protein.   The signal sequence contained in the sequence described in SEQ ID NO: 11 is a kind of leader sequence. The main function of a leader peptide encoded by the leader sequence is presumed to be excretion from inside the cell membrane to outside the cell membrane.   The protein encoded by bases numbers 121 to 1917, namely the portion excluding the leader peptide, is a mature protein and exhibits a high activity of peptide-forming.

Furthermore, the various gene recombination techniques indicated below can be carried out in accordance with the descriptions in Molecular

Cloning, 2nd edition, Cold Spring Harbor Press (1989) and other publications.

A DNA encoding an enzyme that can be used in the present invention can be obtained by polymerase chain reaction (PCR, refer to White, T.J. et al., Trends Genet., 5, 185 (1989)) or hybridization from a chromosomal DNA or a

5   DNA library of *Empedobacter brevis* or *Sphingobacterium* sp.   Primers used in PCR can be designed based on the internal amino acid base sequences determined on the basis of purified peptide-forming enzyme as explained in the previous section (3).   In addition, since the base sequences of the peptide-forming enzyme genes (SEQ ID NO: 5 and SEQ ID NO: 11) have been

10   identified by the present invention, primers or hybridization probes can be designed on the basis of these base sequences, and the gene can be isolated using the probes.   If primers having sequences corresponding to the 5'-nontranslated region and 3'-nontranslated region, respectively, are used as PCR primers, the full-length encoded region of the enzyme can be amplified.

15   Taking as an example the case of amplifying a region containing both the leader sequence and a mature protein encoding region as described in SEQ ID NO: 5, specific examples of primers include a primer having a base sequence of the region upstream of base number 61 in SEQ ID NO: 5 for the 5'-side primer, and a primer having a sequence complementary to the base sequence

20   of the region downstream of base number 1908 for the 3'-side primer.

Primers can be synthesized, for example, according to ordinary methods using the phosphoamidite method (refer to Tetrahedron Letters (1981), 22, 1859) by use of the Model 380B DNA Synthesizer manufactured by Applied Biosystems.   The PCR reaction can be carried out, for example, by

25   using the Gene Amp PCR System 9600 (Perkin-Elmer) and the Takara LA

PCR In Vitro Cloning Kit (Takara Shuzo) in accordance with the method specified by the supplier such as the manufacturer.

A DNA that encodes an enzyme that can be used in the peptide production method of the present invention, regardless of whether the DNA

5   contains a leader sequence or not, includes a DNA that is substantially identical to the DNA consisting of the CDS described in SEQ ID NO: 5 of the Sequence Listing.   Namely, a DNA substantially identical to the DNA of the present invention can be obtained by isolating a DNA consisting of a base sequence complementary to the CDS described in SEQ ID NO: 5 of the

10 Sequence Listing, or a DNA that hybridizes with a probe prepared from the same base sequence under stringent conditions and encodes a protein having peptide-forming activity, from a mutant DNA encoding the enzyme or cells that possess that DNA.

The DNA of the present invention, regardless of whether it contains a

15 leader sequence or not, includes a DNA that is substantially identical to the DNA consisting of the CDS described in SEQ ID NO: 11 of the Sequence Listing.   Namely, a DNA substantially identical to the DNA of the present invention can be obtained by isolating a DNA consisting of a base sequence complementary to the CDS described in SEQ ID NO: 11 of the Sequence

20 Listing, or a DNA that hybridizes with a probe prepared from the same base sequence under stringent conditions and encodes a protein having peptide-forming activity, from a mutant DNA encoding the enzyme or cells that possess that DNA.

Namely, in the present invention, the DNA indicated in (a) to (h) below

25 can be used:

(a) A DNA having a base sequence consisting of bases numbers 127 to 1908 of a base sequence described in SEQ ID NO: 5 of the Sequence Listing;

(b) A DNA that hybridizes with a DNA having a base sequence complementary to the base sequence consisting of bases numbers 127 to

5   1908 of the base sequence described in SEQ ID NO: 5 of the Sequence Listing under stringent conditions, and encodes a protein that has peptide-forming activity;

(c) A DNA having a base sequence consisting of bases numbers 121 to 1917 of a base sequence described in SEQ ID NO: 11 of the Sequence Listing;

10   (d) A DNA that hybridizes with a DNA having a base sequence complementary to the base sequence that consisting of bases numbers 121 to 1917 of the base sequence described in SEQ ID NO: 11 of the Sequence Listing under stringent conditions, and encodes a protein that has peptide-forming activity;

15   (e) A DNA having a base sequence consisting of bases numbers 61 to 1908 of a base sequence described in SEQ ID NO: 5 of the Sequence Listing;

(f) A DNA that hybridizes with a DNA having a base sequence complementary to the base sequence that consisting of bases numbers 61 to 1908 of the base sequence described in SEQ ID NO: 5 of the Sequence Listing

20   under stringent conditions, and encodes a protein that has peptide-forming activity;

(g) A DNA having a base sequence consisting of bases numbers 61 to 1917 of a base sequence described in SEQ ID NO: 11 of the Sequence Listing;

(h) A DNA that hybridizes with a DNA having a base sequence

25   complementary to the base sequence that consisting of bases numbers 61 to

1917 of the base sequence described in SEQ ID NO: 11 of the Sequence Listing under stringent conditions, and encodes a protein containing a mature protein region that has peptide-forming activity.

A probe can be produced in accordance with conventional methods based on, for example, the base sequence described in SEQ ID NO: 5 of the Sequence Listing. In addition, a method for isolating a target DNA by using a probe to find a DNA that hybridizes with the probe may also be carried out in accordance with conventional methods. For example, a DNA probe can be produced by amplifying a base sequence cloned in a plasmid or phage vector, cleaving the base sequence desired to be used as a probe with a restriction enzyme and then extracting the desired base sequence. The cleaving site can be adjusted depending on the target DNA.

The term "stringent conditions" as used herein refers to conditions under which a so-called specific hybrid is formed but no non-specific hybrid is formed. It is difficult to precisely express this condition in numerical values. For example, mention may be made of a condition under which DNAs having a high homology, for example, 50% or more, preferably 80% or more, more preferably 90% or more, hybridize with each other and DNAs having a lower homology than these do not hybridize with each other, or ordinary conditions for rinse in southern hybridization under which hybridization is performed at 60°C in a salt concentration corresponding to 1×SSC and 0.1% SDS, preferably 0.1×SSC, and 0.1% SDS. Although the genes that hybridize under such conditions include genes in which a stop codon occurs at an intermediate site or which lose activity due to a mutation in the active center, these can be easily removed by assaying the enzyme activity of the expression product

using a method to be described later after ligating them to a commercially

available expression vector, expressing them in a suitable host.

However, in the case of a base sequence that hybridizes under

stringent conditions as described above, it is preferable that the protein

5   encoded by that base sequence retains about a half or more, preferably 80%

or more, and more preferably 90% or more, of the enzyme activity of the

protein having the amino acid sequence encoded by the original base

sequence under conditions of 50°C and pH 8.   For example, when explained

on the case of a base sequence that hybridizes under stringent conditions with

10   a DNA that consists of a base sequence complementary to a base sequence

that consists of bases numbers 127 to 1908 of the base sequence described in

SEQ ID NO: 5, it is preferable that the protein encoded by that base sequence

retains about a half or more, preferably 80% or more, and more preferably 90%

or more, of the enzyme activity of the protein having an amino acid sequence

15   that consists of amino acid residues numbers 23 to 616 of the amino acid

sequence described in SEQ ID NO: 6 under conditions of 50°C and pH 8.

An amino acid sequence encoded by the CDS described in SEQ ID

NO: 5 of the Sequence Listing is shown in SEQ ID NO: 6 of the Sequence

Listing.   In addition, an amino acid sequence encoded by the CDS described

20   in SEQ ID NO: 11 of the Sequence Listing is shown in SEQ ID NO: 12 of the

Sequence Listing.   The full-length amino acid sequence described in SEQ ID

NO: 6 contains a leader peptide and a mature protein region, with amino acid

residues numbers 1 to 22 constituting the leader peptide, and amino acid

residues numbers 23 to 616 constituting the mature protein region.   In

25   addition, the full-length amino acid sequence described in SEQ ID NO: 11

includes a leader peptide and a mature protein region, with amino acid residues numbers 1 to 20 constituting the leader peptide, and amino acid residues 21 to 619 constituting the mature protein region.

5 The protein encoded by the DNA of the present invention is a protein in which the mature protein has peptide-forming activity, and a DNA that encodes a protein substantially identical to a protein having the amino acid sequence described in SEQ ID NO: 6 or SEQ ID NO: 12 of the Sequence Listing, regardless of whether it contains a leader peptide or not, is also included in the DNA of the present invention.   (Note that, base sequences are specified from amino acid sequences in accordance with the codes of the universal codons.)
10 Namely, the present invention provides DNAs that encode proteins indicated in (A) to (H):

(A) A protein having an amino acid sequence consisting of amino acid residues numbers 23 to 616 of an amino acid sequence described in SEQ ID
15 NO: 6 of the Sequence Listing;

(B) A protein having an amino acid sequence including substitution, deletion, insertion, addition and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues numbers 23 to 616 of the amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing,
20 and having activity to form, from an amine component that is a peptide being equal to or longer than a dipeptide and a carboxy component, a peptide having one more peptide bond than the amine component;

(C) A protein having an amino acid sequence consisting of amino acid residues numbers 21 to 619 of the amino acid sequence described in SEQ ID
25 NO: 12 of the Sequence Listing;

(D) A protein having an amino acid sequence including substitution, deletion, insertion, addition and/or inversion of one or a plurality of amino acids in the amino acid sequence consisting of amino acid residues numbers 21 to 619 of the amino acid sequence described in SEQ ID NO: 12 of the Sequence Listing, and having activity to form, from an amine component that is a peptide being equal to or longer than a dipeptide and a carboxy component, a peptide having one more peptide bond than the amine component;

(E) A protein having an amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing;

(F) A protein containing a mature protein region, the protein having an amino acid sequence including substitution, deletion, insertion, addition and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing, and having activity to form, from an amine component thati is a peptide being equal to or longer than a dipeptide and a carboxy component, a peptide having one more peptide bond than the amine component;

(G) A protein having an amino acid sequence described in SEQ ID NO: 12 of the Sequence Listing; and,

(H) A protein containing a mature protein region, the protein having an amino acid sequence including substitution, deletion, insertion, addition and/or inversion of one or a plurality of amino acids in the amino acid sequence described in SEQ ID NO: 12 of the Sequence Listing, and having activity to form, from an amine component thati is a peptide being equal to or longer than a dipeptide and a carboxy component, a peptide having one more peptide bond than the amine component.

In the peptide production method of the present invention, one type or two or more types of protein selected from the group consisting of proteins encoded by the DNAs indicated in (a) to (h) above and the group consisting of proteins indicated in (A) to (H) above can be used.

5   Here, the term "a plurality of", which may vary depending on the locations in the three-dimensional structure of the protein of and types of the amino acid residues, means 2 to 100, preferably 2 to 50 and more preferably 2 to 10.   However, in the case of amino acid sequences of the proteins of (B), (D), (F) and (H) containing therein substitution, deletion, insertion, addition

10   and/or inversion of one or a plurality of amino acid residues, it is preferable that the proteins retain about a half or more, more preferably 80% or more, and even more preferably 90% or more of the enzyme activity in comparison with the activity of proteins that contain no mutation under conditions of 50°C and pH 8.   For example, in explaining this on the case of (B) , it is preferable that

15   the protein having the amino acid sequence described in SEQ ID NO: 6 of the Sequence Listing of (B) but contains substitution, deletion, insertion, addition or inversion of one or a plurality of amino acid residues therein retains about a half or more, more preferably 80% or more, and even more preferably 90% or more of the enzyme activity of the protein having the amino acid sequence

20   described in SEQ ID NO: 6 of the Sequence Listing under conditions of 50°C and pH 8.

A mutation of an amino acid such as those indicated in the aforementioned (B) is obtained by modifying the base sequence of the gene of the enzyme of the present invention so that amino acids of the enzyme gene in

25   specific sites are substituted, deleted, inserted or added.   In addition, modified

DNAs such as those described above can also be obtained by conventionally known mutagenesis treatments. Mutagenesis treatments include, for example, a method in which a DNA that encodes the enzyme is treated *in vitro* with hydroxylamine or the like, as well as a method in which bacteria belonging

5   to the genus *Escherichia* that possess a DNA encoding the enzyme are treated by a mutagen normally used in artificial mutagenesis, such as ultraviolet irradiation, N-methyl-N'-nitro-N-nitrosoguanidine (NTG) or nitrous acid.

In addition, the base substitution, deletion, insertion, addition and/or inversion described above also include naturally-occurring mutations such as

10   differences attributable to a microbe species or strain. By expressing a DNA having such a mutation in suitable cells and examining the enzyme activity of the expression product, a DNA can be obtained that encodes the protein described in SEQ ID NO: 6 or 12 of the Sequence Listing or a protein substantially identical thereto.

15   (2-4-2) Construction of Transformants and Production of Peptide-forming Enzymes

A peptide-forming enzyme that can be used in the peptide production method of the present invention can be produced by introducing the DNA explained in the aforementioned section (2-4-1) into a suitable host and

20   expressing within that host.

With respect to hosts for expressing a protein encoded by the DNA, examples of the hosts that can be used include various prokaryotic cells including bacteria belonging to the genus *Escherichia* such as *Escherichia coli*, *Empedobacter*, *Sphingobacterium*, *Flavobacterium* and *Bacillus subtilis*, as

25   well as various eukaryotic cells including *Saccharomyces cerevisiae*, *Pichia*

stipitis and *Aspergillus oryzae.*

A recombinant DNA used to introduce a DNA into a host can be prepared by inserting the DNA to be introduced into a vector corresponding to the type of host in which the DNA is to be expressed, in such a form that the

5   protein encoded by that DNA can be expressed.   If a promoter that is originally associated with the gene encoding the peptide-forming enzyme in, e.g., *Empedobacter brevis* is capable of working in the host cells, such a promoter can be used as the promoter for expressing the DNA in the present invention.   If necessary, another promoter that is capable of working in the

10   host cells may be connected to the DNA of the present invention, for expressing the DNA under the control of the promoter.

Examples of transformation methods for introducing a recombinant DNA into host cells include the method of D.M. Morrison (see Methods in Enzymology, 68, 326 (1979)) or the method in which DNA permeability is

15   increased by treating receptor microbial cells with calcium chloride (see Mandel, H. and Higa, A., J. Mol. Biol., 53, 159 (1970)).

In the case of mass production of a protein using recombinant DNA technology, conjugating the protein within a transformant that produces the protein to form an inclusion body of protein is also a preferable mode for

20   carrying out the present invention.   Advantages of this expression and production method include protection of the target protein from digestion by proteases present within the microbial cells, and simple and easy purification of the target protein by disrupting the microbial cells followed by centrifugal separation and so forth.

25   The inclusion body of protein obtained in this manner is solubilized with

a protein denaturant and the protein is converted to a properly folded, physiologically active protein through an activity regeneration procedure that consists primarily of removal of the denaturant.   There are numerous examples of this, including regeneration of the activity of human interleukin-2

5   (see Japanese Patent Application Laid-open Publication No. S61-257931).

To obtain an active protein from inclusion bodies, a series of procedures including solubilization and activity regeneration are required, and the procedure is more complex than the case of producing the active protein directly.   However, in the case of producing a protein that has a detrimental

10   effect on microbial growth in large volumes within microbial cells, that effect can be suppressed by accumulating the proteins in the form of inclusion bodies of protein that are inactive in the microbial cells.

Examples of mass production methods for producing a target protein in the form of inclusion bodies include a method in which a target protein is

15   expressed independently under the control of a powerful promoter, and a method in which a target protein is expressed in the form of a fused protein with a protein that is known to be expressed in a large volume.

Hereinafter, the present invention will be explained more specifically taking as an example a method for producing transformed *Escherichia coli* and

20   using that transformed microbe to produce a peptide-forming enzyme. Furthermore, in the case of producing peptide-forming enzyme in a microbe such as *Escherichia coli*, a DNA that encodes a precursor protein containing a leader sequence may be used or a DNA that consists only of a mature protein region that does not contain a leader sequence may be used, and the DNA can

25   be suitably selected for the protein encoding sequence depending on the

production conditions, form, usage conditions and so forth of the enzyme to be produced.

Promoters normally used in the production of heterogeneous proteins in *Escherichia coli* can be used as a promoter for expressing a DNA encoding

5   a peptide-forming enzyme. Examples of such promoters include T7 promoter, lac promoter, trp promoter, trc promoter, tac promoter, lambda phage PR promoter, PL promoter and other powerful promoters. In addition, examples of vectors that can be used include pUC19, pUC18, pBR322, pHSG299, pHSG298, pHSG399, pHSG398, RSF1010, pMW119, pMW118, pMW219, and

10   pMW218. Besides, vectors of phage DNA can also be used. Moreover, expression vectors that contain promoters and are capable of expressing an inserted DNA sequence can be used.

To produce peptide-forming enzyme in the form of a fused protein inclusion body, a gene that encodes another protein, and preferably a

15   hydrophilic peptide, is ligated upstream or downstream of the peptide-forming enzyme gene. The gene that encodes another protein in this manner may be any gene that increases the amount of the fused protein accumulated and enhances the solubility of the fused protein after the denaturation and regeneration steps. Examples of candidates for such genes include T7 gene

20   10, β-galactosidase gene, dehydrofolate reductase gene, γ-interferon gene, interleukin-2 gene and prochymosin gene.

When these genes are ligated to the genes that encode peptide-forming enzymes, the genes are ligated so that reading frames of codons are consistent. It is recommended that the genes be ligated at a

25   proper restriction enzyme site or a synthetic DNA having a proper sequence be

utilized.

Further, to increase a production amount of the peptide-forming enzyme, it is preferable in some cases that a terminator, which is a transcription terminating sequence, be ligated to downstream of the fusion

5   protein gene.   The terminator includes, for example, a T7 terminator, an fd phage terminator, a T4 terminator, a tetracycline resistant gene terminator, and an *Escherichia coli* trpA gene terminator.

As the vectors for introducing a gene that encodes a peptide-forming enzyme or a fused protein between the peptide-forming enzyme and another

10   protein in *Escherichia coli* are preferred so-called multi-copy type vectors, examples of which include a plasmid having a replicator derived from ColE1, such as a pUC-based plasmid, and a pBR322-based plasmid or derivatives thereof.   The "derivatives" as used herein refer to those plasmids that are subjected to modification by substitution, deletion, insertion, addition and/or

15   inversion of bases.   Note that the modification as used herein includes modifications by a mutagenesis treatment with a mutagen or UV irradiation, or modifications by spontaneous mutation.

To screen transformants, it is preferable that the vectors have markers such as an ampicillin resistant gene.   These plasmids are commercially

20   available expression vectors having potent promoters (a pUC-based vector (manufactured by Takara Shuzo, Co., Ltd.), such as pRROK-based vector (manufactured by Clonetech Laboratories, Inc.), pKK233-2 (manufactured by Clonetech Laboratories, Inc.) and so forth.

A recombinant DNA is obtained by ligating a DNA fragment to a vector

25   DNA.   In this case, a promoter, a gene encoding the peptide-forming enzyme

or a fused protein consisting of the peptide-forming enzyme and another

protein, and depending on the case, a terminator are ligated in that order.

When *Escherichia coli* is transformed using the recombinant DNA and

the resulting *Escherichia coli* is cultured, a peptide-forming enzyme or a fused

5   protein consisting of the peptide-forming enzyme and another protein is

expressed and produced.   Although a strain that is normally used in the

expression of a heterogeneous gene can be used as a host to be transformed,

Escherichia coli JM109, for example, is preferable.   Methods for

transformation and methods for selection from transformants are described in

10   Molecular Cloning, 2nd Edition, Cold Spring Harbor Press (1989) and other

publications.

In the case of expressing a peptide-forming enzyme in the form of a

fusion protein, the peptide-forming enzyme may be cleaved out using a

restriction protease that uses a sequence not present in the peptide-forming

15   enzyme, such as blood coagulation factor Xa or kallikrein, as the recognition

sequence.

A medium normally used for culturing *Escherichia coli*, such as

M9-casamino acid medium or LB medium, may be used as a production

medium.   In addition, culturing conditions and induction conditions for

20   production are suitably selected according to a variety of the marker of the

vector, promoter, type of host microbe and so forth.

The following method can be used to recover the peptide-forming

enzyme or fused protein consisting of the peptide-forming enzyme and another

protein.   If the peptide-forming enzyme or its fused protein was produced as

25   solubilized form within the microbial cells, after recovering the microbial cells,

the microbial cells are disrupted or lysed so that they can be used as a crude

enzyme liquid. Moreover, the peptide-forming enzyme or its fused protein can

be purified prior to use by ordinary techniques such as precipitation, filtration or

column chromatography as necessary. In this case, a purification method

5   using an antibody of the peptide-forming enzyme or its fused protein can also

be used .

In the case where protein inclusion bodies are formed, the inclusion

bodies are solubilized with a denaturant. They may be solubilized together

with the microbial cell protein. However, in consideration of the following

10   purification procedure, the inclusion bodies are preferably taken out and then

solubilized. Conventionally known methods may be used to recover the

inclusion bodies from the microbial cells. For example, inclusion bodies can

be recovered by disrupting the microbial cells followed by centrifugal

separation. Examples of denaturants capable of solubilizing inclusion bodies

15   include guanidine hydrochloride (for example, 6 M, pH 5 to 8) and urea (for

example, 8 M).

A protein having activity is regenerated by removing these denaturants

by dialysis. A Tris-HCI buffer solution or a phosphate buffer solution and so

forth may be used as solution used in dialysis, and the concentration may be,

20   for example, 20 mM to 0.5 M, while the pH may be, for example, 5 to 8.

The protein concentration during the regeneration step is preferably

held to about 500 μg/ml or less. The dialysis temperature is preferably 5°C or

lower to inhibit the regenerated peptide-forming enzyme from undergoing

self-crosslinking. Moreover, in addition to dialysis, dilution or ultrafiltration

25   may be used to remove the denaturants, and it is expected that the activity can

be regenerated regardless of whichever denaturant is used.

Hereinafter, the present invention will be explained by examples. However, the present invention is not limited to these examples. In addition to confirmation by ninhydrin coloring of thin-film chromatograms (qualitative),

5 quantitative determinations were made by the following high-performance liquid chromatography in order to assay products. Column: InertsiL ODS-2 (manufactured by GL Science, Inc.), eluate: aqueous phosphate solution containing 5.0 mM sodium 1-octanesulfonate (pH 2.1) : methanol = 100 : 15 to 50, flow rate: 1.0 mL/min, detection: 210 nanometers (nm).

10

Examples

(Example 1)   Cultivation of Microbe (*Empedobacter brevis* FERM BP-8113)

A 50 mL medium (pH 6.2) containing 5 grams (g) glucose, 5 g ammonium sulfate, 1 g monopotassium phosphate, 3 g dipotassium phosphate,

15 0.5 g magnesium sulfate, 10 g yeast extract and 10 g peptone in 1 liter (L) was transferred to a 500 mL Sakaguchi flask and sterilized at 115°C for 15 minutes. This medium was then inoculated with one loopful cells of *Empedobacter brevis* strain FERM BP-8113 (Depositary institution: the independent administrative corporation, National Institute of Advanced Industrial Science

20 and Technology, International Patent Organism Depositary, Address of depositary institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date: July 8, 2002) that had been cultured at 30°C for 16 hours in the same medium, followed by shake culturing at 30°C for 16 hours and 120 strokes/min.

25

(Example 2)   Production of Peptide Using Microbial Cells

Microbial cells were collected by centrifuging (10,000 rounds per minute (rpm), 15 minutes) the culture broth obtained in Example 1 followed by suspending to a concentration of 100 g/L in 100 mM borate buffer (pH 9.0) containing 10 mM EDTA.   After respectively adding 1 mL of this suspension to 1 mL of 100 mM borate buffer (pH 9.0) containing 10 mM EDTA, 200 mM of the following carboxy components and 400 mM of the following amino acids to bring to a final volume of 2 mL, the reaction was carried out at 18°C for 2 hours. The peptides that were formed as a result of this reaction are shown in Table 1.

Table 1

Carboxy com-ponent	Amine com-ponent	Formed peptide	(mM)	Carboxy com-ponent	Amine com-pone nt	Formed peptide	(mM)
L-Ala-OMe	L-Leu	L-Ala-L-Leu	38.2	Gly-OMe	L-His	L-Gly-L-His	22.1
	L-Met	L-Ala-L-Met	68.3	L-Ser-OMe	L-Ser	L-Ser-L-Ser	29.0
	L-Phe	L-Ala-L-Phe	62.4	L-Val-OMe	L-Met	L-Val-L-Met	10.5
	L-Ser	L-Ala-L-Ser	51.3	L-Met-OMe	L-Phe	L-Met-L-Phe	28.5
	L-His	L-Ala-L-His	52.1	L-Thr-OMe	L-Leu	L-Thr-L-Leu	23.0
	L-Arg	L-Ala-L-Arg	72.1	L-Ile-OMe	L-Met	L-Ile-L-Met	8.3
	L-Gln	L-Ala-L-Gln	68.0				

(Hydrochloride salts were used for all the carboxy components.)

(Example 3)   Purification of Enzyme

5   The procedure after centrifugal separation was carried out either on ice

or at 4°C. *Empedobacter brevis* strain FERM BP-8113 (Depositary institution:

the independent administrative corporation, National Institute of Advanced

Industrial Science and Technology, International Patent Organism Depositary,

Address of depositary institution: Chuo Dai-6, 1-1 Higashi 1-Chome,

Tsukuba-shi, Ibaraki-ken,, Japan, International deposit transfer date: July 8,

2002) was cultivated in the same manner in as Example 1, and the microbial

cells were collected by centrifugal separation (10,000 rpm, 15 minutes).   After

5   washing 16 g of microbial cells with 50 mM Tris-HCl buffer (pH 8.0), they were

suspended in 40 milliliters (ml or mL) of the same buffer and subjected to

ultrasonic disrupting treatment for 45 minutes at 195 watts.   This sonicate was

then centrifuged (10,000 rpm, 30 minutes) to remove the cell debris and obtain

an ultrasonically disrupted liquid supernatant.   This ultrasonically disrupted

10   liquid supernatant was dialyzed overnight against 50 mM Tris-HCl buffer (pH

8.0) followed by removal of the insoluble fraction by ultracentrifugation (50,000

rpm, 30 minutes) to obtain a soluble fraction in the form of the supernatant

liquid.   The resulting soluble fraction was applied to a Q-Sepharose HP

column (manufactured by Amersham) pre-equilibrated with Tris-HCl buffer (pH

15   8.0), and the active fraction was collected from the non-adsorbed fraction.

This active fraction was dialyzed overnight against 50 mM acetate buffer (pH

4.5) followed by removal of the insoluble fraction by centrifugal separation

(10,000 rpm, 30 minutes) to obtain a dialyzed fraction in the form of the

supernatant liquid.   This dialyzed fraction was then applied to a Mono S

20   column (manufactured by Amersham) pre-equilibrated with 50 mM acetate

buffer (pH 4.5) to elute enzyme with a linear concentration gradient of the same

buffer containing 0 to 1 M NaCl.   The fraction that had the lowest level of

contaminating protein among the active fractions was applied to a Superdex

200pg column (manufactured by Amersham) pre-equilibrated with 50 mM

25   acetate buffer (pH 4.5) containing 1 M NaCl, and gel filtration was performed

by allowing the same buffer (pH 4.5) containing 1 M NaCl to flow through the column to obtain an active fraction solution. As a result of performing these procedures, the peptide-forming enzyme used in the present invention was confirmed to have been purified with homogeneity based on the experimental

5   results of electrophoresis. The enzyme recovery rate in the aforementioned purification process was 12.2% and the degree of purification was 707 folds.

(Example 4)   Measurement of Molecular Weight of Enzyme

     (SDS-Gel Electrophoresis)

10   A 0.3 microgram (μg) equivalent of the purified enzyme fraction obtained by the method of Example 3 was applied to polyacrylamide electrophoresis. 0.3% (w/v) Tris, 1.44% (w/v) glycine and 0.1% (w/v) sodium laurylsulfate were used for the electrophoresis buffer solution, a gel having a concentration gradient of 10 to 20% (Multigel 10 to 20, manufactured by Daiichi

15   Pure Chemicals) was used for the polyacrylamide gel, and Pharmacia molecular weight markers were used for the molecular weight markers. Following completion of electrophoresis, the gel was stained with Coomassie brilliant blue R-250, and a homogeneous band was detected at the location of a molecular weight of about 75 kilodalton (kDa).

20   (Gel filtration)

         The purified enzyme fraction obtained by the method of Example 3 was applied to a Superdex 200pg column (manufactured by Amersham) pre-equilibrated with 50 mM acetate buffer (pH 4.5) containing 1 M NaCl, and gel filtration was carried out by allowing the same buffer (pH 4.5) containing 1

25   M NaCl to flow through the column to measure the molecular weight.

Pharmacia molecular weight markers were used as standard proteins having known molecular weights to prepare a calibration curve.   As a result, the molecular weight of the enzyme was about 150 kDa.

Based on the results of SDS-gel electrophoresis and gel filtration, the enzyme was suggested to be a homodimer having a molecular weight of about 75 kDa.

(Example 5)   Optimum pH of Enzyme

The effects of pH for L-alanyl-L-glutamine production from L-alanine methyl ester hydrochloride and L-glutamine were examined.   Acetate buffer (pH 3.9 to 5.4), MES buffer (pH 5.4 to 6.4), phosphate buffer (pH 6.0 to 7.9), borate buffer (pH 7.8 to 9.3), CAPS buffer (pH 9.3 to 10.7) and K_2HPO_4-NaOH buffer (pH 10.8 to 11.6) were used as buffers.   1 microliter (μl) of the Mono S fraction enzyme obtained in Example 3 (about 180 U/ml) was added to 100 μl of 100 mM each buffer containing 100 mM L-alanine methyl ester, 200 mM L-glutamine and 10 mM EDTA and allowed to react at 18°C for 5 minutes to measure the effects of pH on the reaction.   The results based on assigning a value of 100% to the case of using borate buffer (pH 9.3) are shown in Fig. 1. As a result, the optimum enzyme pH was 8 to 9.5.

(Example 6)   Optimum Temperature of Enzyme

The effects of temperature for L-alanyl-L-glutamine production from L-alanine methyl ester hydrochloride and L-glutamine were examined.   1 μl of the same enzyme fraction used in Example 5 was added to 100 μl of 100 mM borate buffer (pH 9.0) containing 100 mM L-alanine methyl ester, 200 mM

L-glutamine and 10 mM EDTA and allowed to react for 5 minutes at each

temperature to measure the effects of temperature on the reaction.   The

results based on assigning a value of 100% to the activity at 34°C are shown in

Fig. 2.   As a result, the optimum enzyme temperature was 30 to 40°C.

5

(Example 7)   Enzyme Inhibitors

The effects of inhibitors on L-alanyl-L-glutamine production were

examined using L-alanine methyl ester hydrochloride and L-glutamine as

substrates.   2 μl of the same enzyme fraction used in Example 5 was added

10   to 50 μl of 100 mM borate buffer (pH 9.0) containing 10 mM each of the

enzyme inhibitors shown in Table 2, and allowed to react at 25°C for 5 minutes.

Note that, o-phenanthroline, phenylmethylsulfonyl fluoride and

p-nitrophenyl-p'-guanidinobenzoate were dissolved in methanol to a

concentration of 50 mM before use.   The enzyme activity under each

15   condition was indicated as the relative activity in the case of assigning a value

of 100 to the production of L-alanyl-L-glutamine in the absence of enzyme.

inhibitor.   Those results are shown in Table 2.   As a result, among the serine

enzyme inhibitors tested, the enzyme was not inhibited by

phenylmethylsulfonyl fluoride, but it was inhibited by

20   p-nitrophenyl-p'-guanidinobenzoate.

Table 2

	Enzyme inhibitor	Relative activity of L-Ala-L-Gln production (%)
None		100
Metal enzyme inhibitor	EDTA	96
	o-Phenanthroline	96
SH enzyme inhibitor	N-Ethyl maleimide	110
	Monoiodoacetate	101
Serine enzyme inhibitor	Phenylmethylsulfonyl Fluoride	115
	4-(2-Aminoethyl)benzene sulfonyl fluoride	75
	p-Nitrophenyl-p'-guanidino benzoate	0.1

(Example 8)　L-Alanyl-L-Glutamine Production from L-Alanine Methyl Ester and L-Glutamine

　　　　3 μl of the same enzyme fraction as used in Example 5 was added to 100 μl of 100 mM borate buffer (pH 9.0) containing 100 mM L-alanine methyl ester hydrochloride, 200 mM L-glutamine and 10 mM EDTA, and allowed to react at 18°C.　As a result, as shown in Fig. 3, 83 mM L-alanyl-L-glutamine

(L-Ala-L-Gln) was formed in the case of an enzyme-added lot, and the concentration of by-product L-Ala-L-Ala-L-Gln was 1.3 mM. On the other hand, there was scarcely any production of L-Ala-L-Gln observed in an enzyme-non-added lot, and L-Ala-L-Gln production was only about 0.07 mM

5 after reaction for 120 minutes.

(Example 9) Effects of L-Glutamine Concentration on L-Alanyl-L-Glutamine Production

1 μl of the same enzyme fraction as used in Example 5 was added to
10 100 μl of 100 mM borate buffer (pH 9.0) containing 100 mM L-alanine methyl ester hydrochloride, L-glutamine at the concentrations shown in Table 3 and 10 mM EDTA, and allowed to react at 18°C for 2 hours. Those results are shown in Table 3.

Table 3

L-Alanine methyl ester hydrochloride (mM)	L-Glutamine (mM)	L-Ala-L-Gln (mM)
100	100	68.2
	110	72.1
	120	73.3
	130	75.1
	150	75.5
	200	82.0

15

(Example 10) Substrate Specificity of Enzyme (1)

Ester specificity was examined in the case of using L-amino acid ester as the carboxy component. 2 μl of the same enzyme fraction as used in Example 5 was added to 100 μl of 100 mM borate buffer (pH 9.0) containing the 100 mM carboxy components indicated in Table 4, 200 mM L-glutamine

5   and 10 mM EDTA, and allowed to react at 25°C for 2 hours.   The amounts of L-Ala-L-Gln formed in this reaction are shown in Table 4 (HCl represents hydrochloride in Table 4).

Table 4

Carboxy component	L-Ala-L-Gln formed (mM)
L-Alanine methyl ester·HCl	84.3
L-Alanine ethyl ester·HCl	91.5
L-Alanine isopropyl ester·HCl	78.9
L-Alanine-t-butyl ester·HCl	7.5

10   (Example 11) Substrate Specificity of Enzyme (2)

Peptide production by using L-alanine methyl ester as the carboxy component and various L-amino acids as the amine component was examined. 2 μl of the same enzyme fraction as used in Example 5 was added to 100 μl of 100 mM borate buffer (pH 9.0) containing 100 mM L-alanine methyl ester

15   hydrochloride, the 150 mM L-amino acids shown in Table 5 and 10 mM EDTA, and allowed to react at 25°C for 3 hours.   The amounts of each peptide formed in this reaction are shown in Table 5.   (The "+" mark indicates that peptides production was confirmed but were unable to be quantified due to the absence of a standard, while "tr" indicates a trace amount.)

20

Table 5

Amine component	Formed peptide (mM)		Amine component	Formed peptide (mM)	
Gly	L-Ala-Gly	13.7	L-Asn	L-Ala-L-Asn	65.5
L-Ala	L-Ala-L-Ala	25.4	L-Gln	L-Ala-L-Gln	79.3
L-Val	L-Ala-L-Val	20.8	L-Tyr	L-Ala-L-Tyr	17.6
L-Leu	L-Ala-L-Leu	45.3	L-CySH	L-Ala-L-CySH	+
L-Ile	L-Ala-L-Ile	33.9	L-Lys	L-Ala-L-Lys	71.8
L-Met	L-Ala-L-Met	83.3	L-Arg	L-Ala-L-Arg	88.0
L-Phe	L-Ala-L-Phe	74.4	L-His	L-Ala-L-His	66.9
L-Trp	L-Ala-L-Trp	53.9	L-Asp	L-Ala-L-Asp	2.1
L-Ser	L-Ala-L-Ser	62.5	L-Glu	L-Ala-L-Glu	42.9
L-Thr	L-Ala-L-Thr	53.9	L-Pro	L-Ala-L-Pro	tr

(Example 12) Substrate Specificity of Enzyme (3)

Peptide production by using various types of L-amino acid methyl

5   esters as the carboxy component and L-glutamine as the amine component

was examined.   2 μl of the same enzyme fraction as used in Example 5 was

added to 100 μl of 100 mM borate buffer (pH 9.0) containing 100 mM L-amino

acid methyl ester hydrochlorides (AA-OMe·HCl) shown in Table 6, 150 mM

L-glutamine and 10 mM EDTA, and allowed to react at 25°C for 3 hours.   The

10   amounts of each of the peptides formed in this reaction are shown in Table 6.

(The "+" mark indicates that peptides production was confirmed but were

unable to be quantified due to the absence of a standard, while "tr" indicates a

trace amount.) Furthermore, Tween-80 was added to the reaction system to a final concentration of 0.1% in the case of using L-Trp-OMe and L-Tyr-OMe.

Table 6

Carboxy component	Formed peptide (mM)		Carboxy component	Formed peptide (mM)	
Gly-OMe	Gly-L-Gln	54.7	L-Tyr-OMe	L-Tyr-L-Gln	3.4
L-Ala-OMe	L-Ala-L-Gln	74.6	CySH-OMe	L-CySH-L-Gln	+
L-Val-OMe	L-Val-L-Gln	15.4	L-Lys-OMe	L-Lys-L-Gln	+
L-Leu-OMe	L-Leu-L-Gln	+	L-Arg-OMe	L-Arg-L-Gln	7.1
L-Ile-OMe	L-Ile-L-Gln	8.4	L-His-OMe	L-His-L-Gln	+
L-Met-OMe	L-Met-L-Gln	12.0	L-Asp-α-OMe	α-L-Asp-L-Gln	tr
L-Phe-OMe	L-Phe-L-Gln	0.9	L-Asp-β-OMe	β-L-Asp-L-Gln	tr
L-Trp-OMe	L-Trp-L-Gln	+	L-Glu-α-OMe	α-L-Glu-L-Gln	+
L-Ser-OMe	L-Ser-L-Gln	24.0	L-Glu-γ-OMe	γ-L-Glu-L-Gln	+
L-Thr-OMe	L-Thr-L-Gln	81.9	L-Pro-OMe	L-Pro-L-Gln	2.2
L-Asn-OMe	L-Asn-L-Gln	+			
L-Gln-OMe	L-Gln-L-Gln	0.3			

(Hydrochloride salts were used for all of the carboxy components.)

5

(Example 13)　Substrate Specificity of Enzyme (4)

Peptide production by using various L-amino acid methyl esters as the carboxy component and various L-amino acids as the amine component was examined.　2 μl of the same enzyme fraction as used in Example 5 was added

10　to 100 μl of 100 mM borate buffer (pH 9.0) containing 100 mM L-amino acid

methyl ester hydrochlorides (AA-OMe·HCl) shown in Table 7, 150 mM L-amino

acids shown in Table 7 and 10 mM EDTA, and allowed to react at 25°C for 3

hours.   The amounts formed of each of the peptides formed in this reaction

are shown in Table 7.   (The "tr" indicates a trace amount.)   Furthermore,

5   Tween-80 was added to the reaction system to a final concentration of 0.1% in

the case of using L-Trp-OMe.   (The "+" mark indicates that peptides

production was confirmed but were unable to be quantified due to the absence

of a standard.)

Table 7

Carboxy com-ponent	Amine com-ponent	Formed peptide	(mM)	Carboxy com-ponent	Amine com-ponent	Formed peptide	(mM)
Gly-OMe	L-CySH	Gly-L-CySH	45.6	L-Met-OMe	L-Ser	L-Met-L-Ser	12.8
	L-Arg	Gly-L-Arg	25.5		L-Met	L-Met-L-Met	25.0
	L-Phe	Gly-L-Phe	44.0		L-Phe	L-Met-L-Phe	34.0
	L-His	Gly-L-His	31.6	L-Ile-OMe	L-Ser	L-Ile-L-Ser	17.2
	L-Lys	Gly-L-Lys	9.8		L-Met	L-Ile-L-Met	10.0
	L-Ser	Gly-L-Ser	44.2		L-Phe	L-Ile-L-Phe	5.2
L-Thr-OMe	Gly	L-Thr-Gly	9.4	L-Arg-OMe	L-Ser	L-Arg-L-Ser	3.6
	L-Ala	L-Thr-L-Ala	9.4		L-Met	L-Arg-L-Met	0.7
	L-Val	L-Thr-L-Val	0.7		L-Phe	L-Arg-L-Phe	1.9
	L-Leu	L-Thr-L-Leu	28.4	L-Leu-OMe	L-Met	L-Leu-L-Met	12.2
	L-Met	L-Thr-L-Met	38.6	L-Trp-OMe	L-Met	L-Trp-L-Met	4.1
	L-Ser	L-Thr-L-Ser	58.2	L-Lys-OMe	L-Met	L-Lys-L-Met	6.8
L-Ser-OMe	L-Ser	L-Ser-L-Ser	38.0	L-His-OMe	L-Met	L-His-L-Met	6.5
	L-Met	L-Ser-L-Met	12.5	L-Asn-OMe	L-Glu	L-Asn-L-Glu	10.2
	L-Phe	L-Ser-L-Phe	20.3				
L-Val-OMe	L-Ser	L-Val-L-Ser	30.8				
	L-Met	L-Val-L-Met	10.3				
	L-Phe	L-Val-L-Phe	6.1				

(Hydrochloride salts were used for all of the carboxy components.)

(Example 14)   Substrate Specificity of Enzyme (5)

Peptide production by using L or D forms of various amino acid methyl esters as the carboxy component and L or D forms of various amino acids as the amine component was examined.   2 μl of the same enzyme fraction as

5   used in Example 5 was added to 100 μl of 100 mM borate buffer (pH 9.0) containing 100 mM various amino acid methyl ester hydrochlorides (AA-OMe·HCl) shown in Table 8, 150 mM various amino acids shown in Table 8 and 10 mM EDTA, and allowed to react at 25°C for 3 hours.   The amounts of each of the peptides formed in this reaction are shown in Table 8.   (The "tr"

10   indicates a trace amount.)

Table 8

Carboxy component	Amine component	Formed peptide	(mM)
D-Ala-OMe	L-Gln	D-Ala-L-Gln	69.3
D-Ala-OMe	L-Ser	D-Ala-L-Ser	20.3
D-Thr-OMe		D-Thr-L-Ser	1.0
D-Ser-OMe		D-Ser-L-Ser	3.3
D-Val-OMe		D-Val-L-Ser	0.6
D-Met-OMe		D-Met-L-Ser	5.1
L-Ala-OMe	D-Gln	L-Ala-D-Gln	0.3
L-Ala-OMe	D-Ser	L-Ala-D-Ser	5.4
L-Thr-OMe		L-Thr-D-Ser	6.9
L-Ser-OMe		L-Ser-D-Ser	16.2
L-Val-OMe		L-Val-D-Ser	1.4
L-Met-OMe		L-Met-D-Ser	1.9
D-Ala-OMe	D-Gln	D-Ala-D-Gln	tr
D-Ala-OMe	D-Ser	D-Ala-D-Ser	0.2
D-Thr-OMe		D-Thr-D-Ser	1.1
D-Ser-OMe		D-Ser-D-Ser	2.5
D-Val-OMe		D-Val-D-Ser	0.5
D-Met-OMe		D-Met-D-Ser	2.7

(Hydrochloride salts were used for all of the carboxy components.)

(Example 15) Substrate Specificity of Enzyme (6)

Peptide production by using various L-amino acid amides as the carboxy component, and various L-amino acids as the amine component was

5 examined. 2 μl of the same enzyme fraction as that used in Example 5 was added to 100 μl of 100 mM borate buffer (pH 9.0) containing 100 mM L-amino acid amide hydrochlorides (AA-NH_2·HCl) shown in Table 9, 150 mM L-amino acids shown in Table 9 and 10 mM EDTA, and allowed to react at 25°C for 3 hours. The amounts of each of the peptides formed in this reaction are shown

10 in Table 9.

Table 9

Carboxy component	Amine component	Formed Peptide	(mM)
L-Phe-NH_2	L-Gln	L-Phe-L-Gln	0.2
L-Phe-NH_2	L-Ser	L-Phe-L-Ser	0.6
L-Ala-NH_2	L-Gln	L-Ala-L-Gln	7.6
L-Ala-NH_2	L-Met	L-Ala-L-Met	3.4
L-Ala-NH_2	L-His	L-Ala-L-His	3.9
L-Thr-NH_2	L-Gln	L-Thr-L-Gln	0.3

(Hydrochloride salts were used for all of the carboxy components.)

(Example 16) Substrate Specificity of Enzyme (7)

Peptide production by using various L-alanine methyl esters as the

15 carboxy component and C-protected L-amino acids as the amine component was examined. 2 μl of the same enzyme fraction as used in Example 5 was added to 100 μl of 100 mM borate buffer (pH 9.0) containing 100 mM L-alanine

methyl ester hydrochlorides (Ala-OMe·HCl) shown in Table 10, 150 mM

L-amino acid amides shown in Table 10 and 10 mM EDTA, and allowed to

react at 25°C for 3 hours.   The amounts of each of the peptides formed in this

reaction are shown in Table 10.

5

Table 10

Carboxy component	Amine component	Formed peptide	(mM)
L-Ala-OMe	Gly-NH$_2$	L-Ala-Gly-NH$_2$	7.4
	L-Ala-NH$_2$	L-Ala-L-Ala-NH$_2$	8.3
	L-Phe-NH$_2$	L-Ala-L-Phe-NH$_2$	12.2

(Example 17) Substrate Specificity of Enzyme (8)

Peptide production by using various amino acid methyl esters as the

carboxy component and methylamine as the amine component was examined.

10   2 μl of the same enzyme fraction as used in Example 5 was added to 100 μl of

100 mM borate buffer (pH 9.0) containing 100 mM amino acid methyl ester

hydrochlorides (AA-OMe·HCl) shown in Table 11, 150 mM methylamine shown

in Table 11 and 10 mM EDTA, and allowed to react at 25°C for 3 hours.   The

amounts of each of the peptides formed in this reaction are shown in Table 11.

15

Table 11

Carboxy component	Amine component	Formed peptide	(mM)
Gly-OMe		Gly-methylamine	1.1
L-Thr-OMe	Methylamine	L-Thr-methylamine	0.2
L-Ala-OMe		L-Ala-methylamine	0.3

(Hydrochloride salts were used for all of the carboxy components.)

(Example 18) Substrate Specificity of Enzyme (9)

Peptide production by using β-amino acid esters as the carboxy component or β-amino acids as the amine component was examined. 2 μl of the same enzyme fraction as used in Example 5 was added to 100 μl of 100 mM borate buffer (pH 9.0) containing 100 mM carboxy components shown in Table 12 , 150 mM amine components shown in Table 12 and 10 mM EDTA, and allowed to react at 25°C for 3 hours. The amounts of each of the peptides formed in this reaction are shown in Table 12. (The "tr" indicates a trace amount.)

58

Table 12

Carboxy component	Amine component	Formed peptide	(mM)
Gly-OMe	β-Ala	Gly-β-Ala	2.2
Gly-OMe	β-Phe	Gly-β-Phe	0.4
L-Ala-OMe	β-Ala	Ala-β-Ala	7.7
L-Ala-OMe	β-Phe	Ala-β-Phe	1.4
L-Thr-OMe	β-Ala	Thr-β-Ala	3.2
L-Thr-OMe	β-Phe	Thr-β-Phe	1.4
β-Ala-OMe	L-α-Ala	β-Ala-L-α-Ala	tr
β-Ala-OMe	β-Ala	β-Ala-β-Ala	0.2
B-Ala-OMe	L-Gln	β-Ala-L-Gln	0.6
B-Ala-OMe	L-Ser	β-Ala-L-Ser	3.2

(Example 19) Substrate Specificity of Enzyme (10)

Oligopeptide production by using L-amino acid ester as the carboxy component and peptide as the amine component was examined. 2 μl of the same enzyme fraction as used in Example 5 was added to 100 μl of 100 mM borate buffer (pH 9.0) containing 100 mM carboxy components shown in Table 13, 150 mM amine components shown in Table 13 and 10 mM EDTA, and allowed to react at 25°C for 3 hours. The amounts of each of the peptides formed in this reaction are shown in Table 13. As a result, it was clearly demonstrated that the present enzyme can form not only dipeptide, but also long-chain peptides by using a peptide as the amine component.

As has been indicated in the aforementioned Examples 9 to 19, the present enzyme obtained from *Empedobacter brevis* FERM BP-18545 was

determined to have extremely broad substrate specificity.

Table 13

Carboxy component	Amine component	Produced peptide	(mM)
L-Ala-OMe	L-Ala	L-Ala-L-Ala	28.7
	L-Ala-L-Ala	L-Ala-L-Ala-L-Ala	57.5
	L-Ala-L-Ala-L-Ala	L-Ala-L-Ala-L-Ala-L-Ala	44.5
	L-Ala-L-Ala-L-Ala-L-Ala	L-Ala-L-Ala-L-Ala-L-Ala-L-Ala	34.8
	L-Ala-L-Ala-L-Ala-L-Ala-L-Ala	L-Ala-L-Ala-L-Ala-L-Ala-L-Ala-L-Ala	1.4*
	L-Ala-L-Gln	L-Ala-L-Ala-L-Gln	15.2
	Gly-L-Ala	L-Ala-Gly-L-Ala	25.9
	Gly-Gly	L-Ala-Gly-Gly	41.7
	L-His-L-Ala	L-Ala-L-His-L-Ala	55.9
	L-Leu-L-Ala	L-Ala-L-Leu-L-Ala	48.3
	L-Phe-L-Ala	L-Ala-L-Phe-L-Ala	49.7
	L-Phe-Gly	L-Ala-L-Phe-Gly	43.7
Gly-OMe	L-Ala-L-Tyr	Gly-L-Ala-L-Tyr	1.7
	Gly-L-Gln	Gly-Gly-L-Gln	7.2
	Gly-L-Tyr-L-Ala	Gly-Gly-L-Tyr-L-Ala	44.2
L-Thr-OMe	Gly-Gly	L-Thr-Gly-Gly	83.0

(*: Since the solubility of L-Ala-L-Ala-L-Ala-L-Ala-L-Ala was low, the carboxy component was used at a concentration of 10 mM and the amine component was used at 15 mM in this reaction system. The other conditions were the same as those explained in the example.)

(Example 20) Comparison of Ability to Catalyze Peptide Formation with Known Enzymes

The peptide-forming ability of the present enzyme was compared with that of known enzymes. Carboxypeptidase Y described in EP 278787A1 and

the thiol endopeptidases (ficin, papain, bromelain, and chymopapain) described in EP 359399B1 were used for the known enzymes, and they were used in the form of purified enzymes manufactured by Sigma. Homogeneously purified samples were used as the enzyme in Example 3.

5   The enzymes were added to the reaction system.   The amount of the enzyme was shown in Table 14 in terms of the amount of protein.   The reaction was carried out by adding the enzyme to 100 µl of borate buffer (pH 9.0) containing 100 mM L-alanine methyl ester and 200 mM L-glutamine and allowing the resultant to react at 25°C.   Note that the carboxypeptidase used was one

10   dissolved in 10 mM acetate buffer (pH 5.0) containing 1 mM EDTA, while each thiol endopeptidase used was one dissolved in 10 mM acetate buffer (pH 5.0) containing 2 mM EDTA, 0.1 M KCl, and 5 mM dithiothreitol.   The ratios of the production rates of L-alanyl-L-glutamine by these enzymes are shown in Table 14.

15   As a result, the production of an extremely small amount of L-alanyl-L-glutamine was observed even in the absence of enzymes, while a slight increase in the production rate was observed in the carboxypeptidase- or thiol endopeptidase-added lot as compared with the enzyme-non-added lot. In contrast, an overwhelmingly higher rate of production of

20   L-alanyl-L-glutamine was observed in the present enzyme-added lot, and that rate of production was about 5,000 to 100,000 folds higher than those of carboxypeptidase Y and thiol endopeptidases.   As has been described above, the present enzyme was verified to have an extremely high peptide production rate in contrast to any known enzyme.   Furthermore, the present enzyme is a

25   dimer having a molecular weight of about 75,000. In contrast, the

carboxypeptidase Y has been reported to have a molecular weight of about 61,000, while the thiol endopeptidases have been reported to have a molecular weight of about 23,000 to 36,000. Thus, the present enzyme is much higher than the carboxypeptidase Y and the thiol endopeptidase when expressed in

5 an L-alanyl-L-glutamine production rate per molecular weight as compared to when expressed in L-alanyl-L-glutamine production rate per unit weight as indicated in the examples.

Table 14

Enzyme	Amount of enzyme added (protein mg/ml)	L-Ala-L-Gln production rate(mM/min)	Ratio of L-Ala-L-Gln production rate per enzyme unit weight
No enzyme	0	0.0006	
Carboxypeptidase Y	0.61	0.0257	0.0191
Ficin	2.60	0.0096	0.0017
Papain	2.30	0.0106	0.0021
Bromelain	2.80	0.0062	0.0010
Chymopapain	3.60	0.0100	0.0013
Enzyme of present invention	0.02	4.4000	100.0

10 (Example 21) Isolation of Peptide-forming enzyme Gene Derived from *Empedobacter brevis*

Hereinafter, isolation of a peptide-forming enzyme gene will be explained. The microbe used was *Empedobacter brevis* strain FERM BP-8113 (Depositary institution: the independent administrative corporation,

15 National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary, Address of depositary institution: Chuo Dai-6, 1-1

Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit

transfer date: July 8, 2002).   For isolating the gene, *Escherichia coli* JM-109

was used as a host and pUC118 was used as a vector.

(1) Construction of PCR Primer Based on Determined Internal Amino Acid

5   Sequence

A Mixed primer having the base sequences indicated in SEQ ID NO: 3

and SEQ ID NO: 4, respectively, was constructed based on the amino acid

sequences (SEQ ID NOs: 1 and 2) determined by the Edman's decomposition

method detecting a digestion product of a peptide-forming enzyme derived

10   from the *Empedobacter brevis* strain FERM BP-8113 (Depositary institution:

the independent administrative corporation, National Institute of Advanced

Industrial Science and Technology, International Patent Organism Depositary,

Address of depositary institution: Chuo Dai-6, 1-1 Higashi 1-Chome,

Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date: July 8,

15   2002) digested by a lysyl endopeptidase.

(2) Preparation of Microbial Cells

Empedobacter brevis strain FERM BP-8113 (Depositary institution: the

independent administrative corporation, National Institute of Advanced

Industrial Science and Technology, International Patent Organism Depositary,

20   Address of depositary institution: Chuo Dai-6, 1-1 Higashi 1-Chome,

Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date: July 8,

2002) was cultivated at 30°C for 24 hours on a CM2G agar medium (containing

50 g/l glucose, 10 g/l yeast extract, 10 g/l peptone, 5 g/l sodium chloride, and

20 g/l agar, pH 7.0).   One loopful cells of the resulting microbial cells was

25   inoculated into a 500 ml Sakaguchi flask containing 50 ml of a CM2G liquid

medium (the aforementioned medium excluding agar) followed by shaking cultivation at 30°C.

(3) Preparation of Chromosomal DNA from Microbial Cells

First, 50 ml of culture broth was centrifuged (12,000 rpm, 4°C, 15

5 minutes) to collect the microbial cells. Then, a chromosomal DNA was obtained from the microbial cells using the QIAGEN Genomic-Tip System (Qiagen) based on the procedure described in the manual therefor.

(4) Preparation of DNA Fragment Containing a Portion of Gene for Peptide-forming Enzyme by PCR

10 A DNA fragment containing a portion of gene for the peptide-forming enzyme derived from *Empedobacter brevis* strain FERM BP-8113 (Depositary institution: the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary, Address of depositary institution: Chuo Dai-6, 1-1 Higashi

15 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date: July 8, 2002) was obtained by the PCR method using LA-Taq (manufactured by Takara Shuzo). A PCR reaction was then carried out by using the primers having the base sequences of SEQ ID NOs: 3 and 4 to a chromosomal DNA obtained from *Empedobacter brevis* strain FERM BP-8113 (Depositary

20 institution: the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary, Address of depositary institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date: July 8, 2002).

25 The PCR reaction was carried out for 30 cycles under the following

conditions using Takara PCR Thermal Cycler PERSONAL (manufactured by Takara Shuzo).

94°C   30 seconds

52°C   1 minute

5   72°C   1 minute

After the reaction, 3 μl of the reaction mixture was applied to 0.8% agarose electrophoresis.   As a result, it was verified that a DNA fragment of about 1.5 kilobases (kb) was amplified.

(5) Cloning of Gene for Peptide-Forming Enzyme from Gene Library

10   In order to obtain the gene for peptide-forming enzyme in full-length, southern hybridization was carried out by using the DNA fragment amplified in the PCR procedure as a probe.   The procedure for southern hybridization is explained in Molecular Cloning, 2nd edition, Cold Spring Harbor Press (1989).

The approximately 1.5 kb DNA fragment amplified by the PCR

15   procedure was separated by 0.8% agarose electrophoresis.   The target band was then cut out and purified.   The DNA fragment was labeled with digoxinigen as probe by using DIG High Prime (manufactured by Boehringer-Mannheim) based on the procedure described in the manual therefor.

20   After completely digesting the chromosomal DNA of *Empedobacter brevis* obtained in the step (3) of the present Example 21 by reacting at 37°C for 16 hours with restriction enzyme HindIII, the resultant was electrophoresed on 0.8% agarose gel.   The electrophoresed chromosomal DNA was blotted onto a positively charged Nylon membrane filter (manufactured by Roche

25   Diagnostics) from the agarose gel after the electrophoresis, followed by

treatments of alkali denaturation, neutralization and immobilization. Hybridization was carried out by using EASY HYB (manufactured by Boehringer-Mannheim). After pre-hybridizing the filter at 50°C for 1 hour, the probe labeled with digoxinigen prepared as described above was added and

5   hybridization was carried out at 50°C for 16 hours. Subsequently, the filter was washed for 20 minutes at room temperature with 2 × SSC containing 0.1% SDS. Moreover, the filter was additionally washed twice at 65°C for 15 minutes with 1 × SSC.

Detection of bands that hybridized with the probe was carried out by

10 using the DIG Nucleotide Detection Kit (manufactured by Boehringer-Mannheim) based on the procedure described in the manual therefor. As a result, an about 4 kb band was able to be detected that hybridized with the probe.

Then, 5 μg of the chromosomal DNA prepared in the step (3) of the

15 present Example 21 was completely digested with HindIII. An about 4 kb DNA was separated by 0.8% agarose gel electrophoresis, followed by purification of the DNA using the Gene Clean II Kit (manufactured by Funakoshi) and dissolving the DNA in 10 μl of TE. 4 μl of this product was then mixed with pUC118 HindIII/BAP (manufactured by Takara Shuzo) and a

20 ligation reaction was carried out by using the DNA Ligation Kit Ver. 2 (manufactured by Takara Shuzo). 5 μl of the ligation reaction mixture and 100 μl of competent cells of *Escherichia coli* JM109 (manufactured by Toyobo) were mixed to transform the *Escherichia coli*. This was then applied to a suitable solid medium to construct a chromosomal DNA library.

25 To obtain the full-length gene for peptide-forming enzyme, the

chromosomal DNA library was screened by colony hybridization using the aforementioned probe.   The procedure for colony hybridization is explained in Molecular Cloning, 2nd edition, Cold Spring Harbor Press (1989).

The colonies of the chromosomal DNA library were transferred on a

5   Nylon membrane filter (Nylon Membrane for Colony and Plaque Hybridization (manufactured by Roche Diagnostics) followed by treatments of alkali denaturation, neutralization and immobilization.   Hybridization was carried out by using EASY HYB (manufactured by Boehringer-Mannheim).   After pre-hybridizing the filter at 37°C for 1 hour, the aforementioned probe labeled

10   with digoxinigen was added, followed by hybridization at 50°C for 16 hours. Subsequently, the filter was washed for 20 minutes at room temperature with 2 × SSC containing 0.1% SDS.   In addition, the filter was additionally washed twice at 65°C for 15 minutes with 1 × SSC.

Detection of colonies hybridizing with the labeled probe was carried out

15   by using the DIG Nucleotide Detection Kit (manufactured by Boehringer-Mannheim) based on the explanation described in the manual therefor.   As a result, two strains of colonies were verified to hybridize with the labeled probe.

(6) Base Sequence of Gene for Peptide-forming enzyme Derived from

20   *Empedobacter brevis*

Plasmids possessed by *Escherichia coli* JM109 were prepared from the aforementioned two strains of microbial cells that were verified to hybridize with the labeled probe by using the Wizard Plus Minipreps DNA Purification System (manufactured by Promega) and the base sequence of a portion where

25   hybridization with the probe occurred and nearby was determined.   The

sequencing reaction was carried out by using the CEQ DTCS-Quick Start Kit (manufactured by Beckman-Coulter) based on the procedure described in the manual therefor.   In addition, electrophoresis was carried out by using the CEQ 2000-XL (manufactured by Beckman-Coulter).

5   As a result, it was verified that an open reading frame that encodes a protein containing the internal amino acid sequences of the peptide-forming enzyme (SEQ ID NOs: 1 and 2) did exist, and that the open reading frame was a gene encoding the peptide-forming enzyme.   The base sequence of the full-length gene for peptide-forming enzyme along with the corresponding

10   amino acid sequences is shown in SEQ ID NO: 5.   As a result of analysis on the homology of the resulting open reading frame with the BLASTP program, homology was discovered with two enzymes; it showed a homology of 34% as amino acid sequence with the a-amino acid ester hydrolase of *Acetobacter pasteurianus* (see Appl. Environ. Microbiol., 68(1), 211-218 (2002), and a

15   homology of 26% as amino acid sequence with the glutaryl-7ACA acylase of *Brevibacillus laterosporum* (see J. Bacteriol., 173(24), 7848-7855 (1991).

(Example 22)   Expression of Gene for Peptide-forming Enzyme Derived from *Empedobacter brevis* in *Escherichia coli*

20   The promoter region of the trp operon on the chromosomal DNA of *Escherichia coli* W3110 was amplified by PCR using the oligonucleotides indicated in SEQ ID NOs: 7 and 8 as primers, and the resulting DNA fragments were ligated to a pGEM-Teasy vector (manufactured by Promega). *Escherichia coli* JM109 was then transformed with this ligation solution, and

25   those strains having the target plasmid in which the direction of the inserted trp

promoter is opposite to the orientation of the lac promoter were selected from ampicillin-resistant strains. Next, a DNA fragment containing the trp promoter obtained by treating this plasmid with EcoO109I/EcoRI was ligated to an EcoO109I/EcoRI treatment product of pUC19 (manufactured by Takara).

5 Escherichia coli JM109 was then transformed with this ligation solution and those strains having the target plasmid were selected from ampicillin-resistant strains. Next, a DNA fragment obtained by treating this plasmid with HindIII/PvuII was ligated to a DNA fragment containing an rrnB terminator obtained by treating pKK223-3 (manufactured by Amersham Pharmacia) with

10 HindIII/HincII. Escherichia coli JM109 was then transformed with this ligation solution, strains having the target plasmid were selected from ampicillin-resistant strains, and the plasmid was designated as pTrpT.

The target gene was amplified by PCR using the chromosomal DNA of Empedobacter brevis strain FERM BP-8113 (Depositary institution: the

15 independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary, Address of depositary institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date: July 8, 2002) as a template and the oligonucleotides indicated in SEQ ID NOs: 9 and

20 10 as primers. This DNA fragment was then treated with NdeI/PstI, and the resulting DNA fragment was ligated with the NdeI/PstI treatment product of pTrpT. Escherichia coli JM109 was then transformed with this ligation solution, those strains having the target plasmid were selected from ampicillin-resistant strains, and this plasmid was designated as pTrpT_Gtg2.

25 Escherichia coli JM109 having pTrpT_Gtg2 was seed cultured at 30°C

for 24 hours in LB medium containing 100 mg/l of ampicillin. 1 ml of the resulting culture broth was transferred in a 500 ml Sakaguchi flask containing 50 ml of a medium (D-glucose 2 g/l, yeast extract 10 g/l, casamino acid 10 g/l, ammonium sulfate 5 g/l, potassium dihydrogen phosphate 3 g/l, dipotassium

5 hydrogen phosphate 1 g/l, magnesium sulfate heptahydrate 0.5 g/l, and ampicillin 100 mg/l), followed by cultivation at 25°C for 24 hours. The culture broth had an L-alanyl-L-glutamine production activity of 0.44 U per ml of culture broth and it was verified that the cloned gene was expressed by *Escherichia coli*. Furthermore, no activity was detected for a transformant in which only

10 pTrpT had been introduced as a control.

(Prediction of Signal Sequence)

When the amino acid sequence of SEQ ID NO: 6 described in the Sequence Listing was analyzed with the Signal P v 1.1 program (see Protein Engineering, Vol. 12, No. 1, pp. 3-9, 1999), it was predicted that numbers 1 to

15 22 in amino acid sequences was operated as a signal to secrete into the periplasm, while the mature protein was estimated to be downstream of amino acid number 23.

(Verification of Secretion)

Escherichia coli JM109, having pTrpT_Gtg2, was seed cultured at

20 30°C for 24 hours in LB medium containing 100 mg/l of ampicillin. 1 ml of the resulting culture broth was transferred into a 500 ml Sakaguchi flask containing 50 ml of medium (glucose 2 g/l, yeast extract 10 g/l, casamino acid 10 g/l, ammonium sulfate 5 g/l, potassium dihydrogen phosphate 3 g/l, dipotassium hydrogen phosphate 1 g/l, magnesium sulfate heptahydrate 0.5 g/l, and

25 ampicillin 100 mg/l), followed by final cultivation at 25°C for 24 hours to obtain

cultured microbial cells.

The cultured microbial cells were fractionated into a periplasm fraction and a cytoplasm fraction by an osmotic pressure shock method using a 20 grams/deciliter (g/dl) sucrose solution. The microbial cells immersed in the 20

5   g/dl sucrose solution were immersed in a 5 mM aqueous $MgSO_4$ solution. The centrifuged supernatant was named a periplasm fraction (Pe). In addition, the centrifuged sediment was re-suspended and subjected to ultrasonic disrupting. The resultant was named a cytoplasm fraction (Cy). The activity of glucose 6-phosphate dehydrogenase, which is known to be present in the

10 cytoplasm, was used as an indicator to verify that the cytoplasm had been separated. This measurement was carried out by adding a suitable amount of enzyme to a reaction mixture at 30°C containing 1 mM glucose 6-phosphate, 0.4 mM NADP, 10 mM $MgSO_4$, and 50 mM Tris-Cl (pH 8), followed by measurement of absorbance at 340 nm to measure production of NADPH.

15 The amounts of enzymes in the periplasm fraction and the cytoplasm fraction when the activity of a separately prepared cell-free extract was assigned a value of 100% are shown in Fig. 4. That glucose 6-phosphate dehydrogenase activity did not contain in the periplasm fraction indicates that the periplasm fraction did not mix with the cytoplasm fraction. About 60% of

20 the Ala-Gln production activity was recovered in the periplasm fraction, and it was verified that the Ala-Gln-forming enzyme was secreted into the periplasm as predicted from the amino acid sequence using the Signal P v 1.1 program.

(Example 23) Substrate Specificity of Enzyme (11)

25 Using an enzyme fraction having an Ala-Gln-producing activity

prepared from *Empedobacter brevis* strain FERM BP-8113 (Depositary

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5   1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date:

July 8, 2002), the substrate specificity thereof was further examined.   A 100 μl

reaction mixture consisting of 100 mM borate buffer (pH 9.0) containing various

carboxy components and amine components at the final concentrations shown

in Table 15, enzyme (0.1 units added to the reaction mixture), and 10 mM

10   EDTA were allowed to react at 25°C for the reaction times shown in Table 15.

The production amounts of each of the peptides produced in this reaction are

shown in Table 15.   (The "+" mark indicates that peptides production was

confirmed but were unable to be quantified due to the absence of a standard

preparation, while "tr" indicates a trace amount.)

15

Table 15

Carboxy component (mM)		Amine component (mM)		Produced peptide (mM)		Reaction time (hr)
H-Ala-OMe	50mM	H-p-F-Phe-OH	50mM	H-Ala-p-F-Phe-OH	21.9mM	3
H-Ala-OMe	40mM	H-Cl-F-Phe-OH	40mM	H-Ala-Cl-F-Phe-OH	20.8mM	3
H-Ala-OMe	40mM	H-p-NO$_2$-Phe-OH	40mM	H-Ala-p-NO$_2$-Phe-OH	27.5mM	3
H-Ala-OMe	100mM	H-t-Leu-OH	150mM	H-Ala-t-Leu-OH	0.4mM	3
H-Ala-OMe	20mM	H-2-Nal-OH	20mM	H-Ala-2-Nal-OH	+	3
H-p-F-Phe-OMe	100mM	H-Gln-OH	150mM	H-p-F-Phe-H-Gln-OH	tr	3
H-Cl-F-Phe-OMe	25mM	H-Gln-OH	50mM	H-Cl-F-Phe-H-Gln-OH	tr	3
H-p-NO$_2$-Phe-OMe	40mM	H-Gln-OH	40mM	H-p-NO$_2$-Phe-H-Gln-OH	1.1mM	3
H-t-Leu-OMe	100mM	H-Gln-OH	150mM	H-t-Leu-H-Gln-OH	tr	3
H-2-Nal-OMe	40mM	H-Gln-OH	40mM	H-2-Nal-H-Gln-OH	tr	3
H-Aib-OMe	100mM	H-Gln-OH	150mM	H-Aib-H-Gln-OH	18.8mM	3
H-N-Me-Ala-OMe	100mM	H-Gln-OH	150mM	H-N-Me-Ala-H-Gln-OH	0.5mM	3
H-Aib-OMe	100mM	H-Phe-OH	150mM	H-Aib-Phe-OH	17.2mM	3
H-CHA-OMe	40mM	H-Phe-OH	40mM	H-CHA-Phe-OH	+	3
H-N-Me-Ala-OMe	100mM	H-Phe-OH	150mM	H-N-Me-Ala-Phe-OH	tr	3
H-Ala-OMe	100mM	H-Ser(tBu)-OH	150mM	H-Ala-Ser(tBu)-OH	48.8mM	2
H-Ser(tBu)-OMe	100mM	H-Gln-OH	150mM	H-Ser(tBu)-Gln-OH	tr	2
H-Ala-OMe	100mM	H-Asp(OtBu)-OH	150mM	H-Ala-Asp(OtBu)-OH	62.6mM	2
H-Asp(OtBu)-OMe	100mM	H-Gln-OH	150mM	H-Asp(OtBu)-Gln-OH	0.9mM	2
H-Ala-OMe	100mM	H-Lys(Boc)-OH	150mM	H-Ala-Lys(Boc)-OH	51.0mM	2
H-Lys(Boc)-OMe	100mM	H-Gln-OH	150mM	H-Lys(Boc)-Gln-OH	+	2

Explanation of abbreviations;

H-Ala-OMe: L-Alanine methyl ester hydrochloride

5   H-p-F-Phe-OMe: p-Fluoro-L-phenylalanine methyl ester hydrochloride

H-Cl-F-Phe-OMe: p-Chloro-L-phenylalanine methyl ester hydrochloride

H-p-NO$_2$-Phe-OMe: p-Nitro-L-phenylalanine methyl ester hydrochloride

H-t-Leu-OMe: tert-L-Leucine methyl ester hydrochloride

H-2-Nal-OMe: 3-(2-Naphthyl)-L-alanine methyl ester hydrochloride

H-Aib-OMe: α-Aminoisobutyric acid methyl ester hydrochloride

H-N-Me-Ala-OMe: N-Methyl-L-alanine methyl ester hydrochloride

H-CHA-OMe: β-Cyclohexyl-L-alanine methyl ester hydrochloride

H-Ser(tBu)-OMe: O-tert-Butyl-L-serine methyl ester hydrochloride

5 H-Asp(OtBu)-OMe: L-Aspartic acid β-tert-butyl ester α-methyl ester

hydrochloride

H-Lys(Boc)-OMe: N-ε-tert-Butoxycarbonyl-L-lysine methyl ester hydrochloride

H-p-F-Phe-OH: p-Fluoro-L-phenylalanine

H-Cl-F-Phe-OH: p-Chloro-L-phenylalanine

10 H-p-NO$_2$-Phe-OH: p-Nitro-L-phenylalanine

H-t-Leu-OH: tert-L-Leucine

H-2-Nal-OH: 3-(2-Naphthyl)-L-alanine

H-Gln-OH: L-Glutamine

H-Phe-OH: L-Phenylalanine

15 H-Ser(tBu)-OH: O-tert-Butyl-L-serine

H-Asp(OtBu)-OH: L-Aspartic acid β-tert-butyl ester

H-Lys(Boc)-OH: N-ε-tert-Butoxycarbonyl-L-lysine

(Example 24) Substrate Specificity of Enzyme (12)

20   Using the same enzyme fraction as that in Example 23, substrate

specificity with respect to oligopeptide production was examined.   100 μl of a

reaction mixture consisting of a 10 mM EDTA-containing100 mM borate buffer

(pH 9.0) containing various carboxy components and amine components at the

final concentrations shown in Table 16, and an enzyme (unit numbers added to

25 the reaction mixture are described in Table 18) were allowed to react at 25°C

for 3 hours.   The amounts of each kind of the oligopeptides produced in this reaction are shown in Table 18 (the "+" mark indicates that peptides production was confirmed but were unable to be quantified due to the absence of standard preparations, while "tr" indicates a trace amount).   Furthermore,

5   hydrochlorides were used for all the carboxy components.

Table 16

Carboxy component	Amine component	Amount of enzyme	Produced peptide (mM)	
Gly-OMe	L-Phe-L-Met	1.0	Gly-Phe-Met	13.3
L-Ala-OMe	L-Phe-L-Met	0.2	L-Ala-L-Phe-L-Met	+
L-Tyr-OMe	Gly-Gly-L-Phe-L-Met	1.0	L-Tyr-Gly-Gly-L-Phe-L-Met	2.7
L-Ala-OMe	Gly-Gly-L-Phe-L-Met	0.2	L-Ala-Gly-Gly-L-Phe-L-Met	+
Gly-OMe	Gly-L-Phe	0.1	Gly-L-Phe	17.3
L-Ala-OMe	Gly-L-Phe	0.1	L-Ala-Gly-L-Phe	+
D-Ala-OMe	Gly-L-Phe	0.1	D-Ala-Gly-L-Phe	Tr

(Example 25) Production of L-Alanyl-L-Glutamine Using Microbial Cells of *Sphingobacterium* sp.

10   A 50 ml medium (pH 7.0) containing 5 g glucose, 5 g ammonium sulfate, 1 g monopotassium phosphate, 3 g dipotassium phosphate, 0.5 g magnesium sulfate, 10 g yeast extract and 10 g peptone in 1 L was transferred to a 500 mL Sakaguchi flask and sterilized at 115°C for 15 minutes for culturing *Sphingobacterium* sp. strain FERM BP-8124 (Depositary institution: the

15   independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary, Address of depositary institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit date: July 22, 2002).

This was then inoculated with one loopful cells of *Sphingobacterium* sp. strain

FERM BP-8124 (Depositary institution: the independent administrative

corporation, National Institute of Advanced Industrial Science and Technology,

International Patent Organism Depositary, Address of depositary institution:

5   Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan,

International deposit date: July 22, 2002) cultivated at 30°C for 24 hours on a

slant agar medium (pH 7.0) containing 5 g glucose, 10 g yeast extract, 10 g

peptone and 5 g NaCl and 20 g/L agar in 1 L, followed by shaking cultivation at

30°C for 20 hours and 120 strokes/minute.   1 ml of this culture broth was then

10   added to the aforementioned medium (50 ml/500 mL Sakaguchi flask) and

cultivated at 30°C for 18 hours.   After the cultivation, the microbial cells were

separated from the culture broth by centrifugation and suspended in 0.1 M

borate buffer (pH 9.0) containing 10 mM EDTA to 100 g/L cells as wet basis.

0.1 mL of 100 mM borate buffer (pH 9.0) containing 10 mM EDTA, 200 mM

15   L-alanyl methyl ester hydrochloride and 400 mM L-glutamine was then added

to 0.1 mL of this microbial cell suspension to bring a final volume 0.2 mL, was

allowed to react at 25°C for 120 minutes.   The amount of L-alanyl-L-glutamine

formed was 62 mM.

20   (Example 26) Purification of Enzyme from *Sphingobacterium* sp.

The following procedure after centrifugal separation was carried out

either on ice or at 4°C.   *Sphingobacterium* sp. FERM BP-8124 (Depositary

institution: the independent administrative corporation, National Institute of

Advanced Industrial Science and Technology, International Patent Organism

25   Depositary, Address of depositary institution: Chuo Dai-6, 1-1 Higashi

1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit date: July 22, 2002) was cultivated in the same manner as Example 25, and the microbial cells were collected by centrifugal separation (10,000 rpm, 15 minutes). After washing 2 g of microbial cells with 20 mM Tris-HCl buffer (pH 7.6), they were

5   suspended in 8 ml of the same buffer and subjected to ultrasonic disrupting treatment for 45 minutes at 195 W. This ultrasonically disrupted solution was then centrifuged (10,000 rpm, 30 minutes) to remove the cell debris and obtain an ultrasonically disrupted supernatant. This supernatant was dialyzed overnight against 20 mM Tris-HCl buffer (pH 7.6) followed by removal of the

10   insoluble fraction by ultracentrifugation (50,000 rpm, 30 minutes) to obtain a soluble fraction in the form of the supernatant solution. The resulting soluble fraction was applied to a Q-Sepharose HP column (manufactured by Amersham) pre-equilibrated with Tris-HCl buffer (pH 7.6), and the active fraction was collected from the non-adsorbed fraction. This active fraction

15   was dialyzed overnight against 20 mM acetate buffer (pH 5.0) followed by removal of the insoluble fraction by centrifugal separation (10,000 rpm, 30 minutes) to obtain a dialyzed fraction in the form of the supernatant solution. This supernatant was then applied to an SP-Sepharose HP column (manufactured by Amersham) pre-equilibrated with 20 mM acetate buffer (pH

20   5.0) to obtain the active fraction in which enzyme was eluted with a linear concentration gradient of the same buffer containing 0 to1 M NaCl.

(Example 27) Production of L-Alanyl-L-Glutamine Using Enzyme Fraction

10 µl of the SP-Sepharose HP fraction (about 27 U/ml) purified in

25   Example 26 was added to 90 µl of 111 mM borate buffer (pH 9.0) containing

111 mM L-alanine methyl ester hydrochloride, 222 mM L-glutamine and 11 mM

EDTA, and allowed to react at 25°C for 120 minutes.   As a result, 73 mM of

L-alanyl-L-glutamine was formed in the enzyme-added lot.   On the other hand,

there was scarcely any production of L-Ala-L-Glu observed in the

5   enzyme-non-added lot, and the production amount was only about 0.07 mM

after reaction for 120 minutes.

(Example 28) Substrate Specificity of Enzyme (13)

Substrate specificity of the enzyme from Sphingobacterium sp. strain

10   FERM BP-8124 (Depositary institution: the independent administrative

corporation, National Institute of Advanced Industrial Science and Technology,

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Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan,

International deposit date: July 22, 2002) was examined.   100 μl of 100 mM

15   borate buffer (pH 9.0) containing the various carboxy components at a final

concentration of 100 mM and the various amine components at a final

concentration of 150 mM shown in Tables 17-1 to 17-4, the SP-Sepharose HP

fraction enzyme purified in Example 26 (addition of 0.33 units in the reaction

mixture) and EDTA at a final concentration of 10 mM were allowed to react at

20   25°C for 1.5 hours.   The amounts of each of the peptides formed in this

reaction are shown in Table 17.   (The "+" mark indicates that peptides

production was confirmed but were unable to be quantified due to the absence

of a standard, while "tr" indicates a trace amount.)   Furthermore, Tween-80

was added to the reaction mixture to a final concentration of 0.1% in the case

25   of using L-Tyr-OMe.   In addition, hydrochlorides were used for all carboxy

components.

Table 17-1

Carboxy component	Amine component	Produced peptide (mM)	
L-Ala-OMe	Gly	L-Ala-Gly	11.1
	L-Ala	L-Ala-L-Ala	13.1
	L-Val	L-Ala-L-Val	10.9
	L-Leu	L-Ala-L-Leu	33.0
	L-Ile	L-Ala-L-Ile	24.7
	L-Met	L-Ala-L-Met	86.9
	L-Pro	L-Ala-L-Pro	1.5
	L-Phe	L-Ala-L-Phe	69.5
	L-Trp	L-Ala-L-Trp	46.0
	L-Thr	L-Ala-L-Thr	47.3
	L-Asn	L-Ala-L-Asn	52.3
	L-Tyr	L-Ala-L-Tyr	11.1
	L-CySH	L-Ala-L-CySH	+
	L-Lys	L-Ala-L-Lys	71.2
	L-Arg	L-Ala-L-Arg	72.2
	L-His	L-Ala-L-His	73.6
	L-Asp	L-Ala-L-Asp	2.3
	L-Glu	L-Ala-L-Glu	39.1
	L-Ser	L-Ala-L-Ser	43.8
	D-Ser	L-Ala-D-Ser	3.3
D-Ala-OMe	L-Ser	D-Ala-L-Ser	24.1
	D-Ser	D-Ala-D-Ser	5.5

Table 17-2

Carboxy component	Amine component	Produced peptide (mM)	
L-Thr-OMe		L-Thr-L-Gln	36.1
Gly-OMe		Gly-L-Gln	61.1
L-Ser-OMe		L-Ser-L-Gln	12.9
L-Val-OMe		L-Val-L-Gln	8.2
L-Met-OMe		L-Met-L-Gln	32.6
L-Ile-OMe	L-Gln	L-Ile-L-Gln	6.4
L-Arg-OMe		L-Arg-L-Gln	17.2
L-Tyr-OMe		L-Tyr-L-Gln	0.6
L-Pro-OMe		L-Pro-L-Gln	1.8
L-Phe-OMe		L-Phe-L-Gln	0.8
L-Gln-OMe		L-Gln-L-Gln	0.1
Asp-α-OMe		α-L-Asp-L-Gln	0.05

Table 17-3

Carboxy component	Amine component	Produced peptide (mM)	
L-Thr-OMe	Gly	L-Thr-Gly	0.4
	L-Ala	L-Thr-L-Ala	5.8
	L-Val	L-Thr-L-Val	1.3
	L-Leu	L-Thr-L-Leu	15.3
	L-Met	L-Thr-L-Met	28.9
Gly-OMe	L-Arg	Gly-L-Arg	17.9
	L-Phe	Gly-L-Phe	20.0
	L-His	Gly-L-His	36.2
	L-Lys	Gly-L-Lys	48.2
	L-Ser	Gly-L-Ser	53.8
L-Ser-OMe	L-Ser	L-Ser-L-Ser	9.9
	L-Met	L-Ser-L-Met	7.6
	L-Phe	L-Ser-L-Phe	4.3
L-Val-OMe	L-Ser	L-Val-L-Ser	31.9
	L-Met	L-Val-L-Met	6.8
	L-Phe	L-Val-L-Phe	1.0
L-Met-OMe	L-Ser	L-Met-L-Ser	25.3
	L-Met	L-Met-L-Met	28.4
	L-Phe	L-Met-L-Phe	8.9
L-Ile-OMe	L-Ser	L-Ile-L-Ser	17.3
	L-Met	L-Ile-L-Met	5.1
	L-Phe	L-Ile-L-Phe	1.5
L-Arg-OMe	L-Ser	L-Arg-L-Ser	2.2
	L-Met	L-Arg-L-Met	tr
	L-Phe	L-Arg-L-Phe	tr

Table 17-4

Carboxy component	Amine component	Produced peptide (mM)	
L-Ala-OMe	Gly amide	L-Ala-Gly amide	15.1
	L-Ala amide	L-Ala-L-Ala amide	9.2
	L-Phe amide	L-Ala-Phe amide	27.1
L-Ala-OMe	methylamine	L-Ala-methylamine	0.6
L-Thr-OMe		L-Thr-methylamine	0.3
Gly-OMe		Gly-methylamine	1.0
L-Ala amide	L-Gln	L-Ala-L-Gln	0.3
	L-Met	L-Ala-L-Met	tr
	L-His	L-Ala-L-His	tr

(Example 29) Substrate Specificity of Enzyme (14)

Substrate specificity with respect to oligopeptide production was examined for enzyme derived from *Sphingobacterium* sp. strain FERM BP-8124 (Depositary institution: the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary, Address of depositary institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit date: July 22, 2002).   100 μl of 100 mM borate buffer (pH 9.0) containing the various carboxy components at a final concentration of 100 mM and the various amine components at a final concentration of 150 mM shown in Table 18, the SP-Sepharose HP fraction enzyme purified in Example 26 (addition of 0.33 units in the reaction mixture) and EDTA at a final concentration of 10 mM

were allowed to react for 1.5 hours at 25°C. The amounts of each oligopeptide formed in this reaction are shown in Table 18. Note that hydrochlorides were used for all carboxy components.

Table 18

Carboxy component	Amine component	Produced peptide (mM)	
L-Ala-OMe	L-Ala	L-Ala-L-Ala	25.6
	L-Ala-L-Ala	L-Ala-L-Ala-L-Ala	41.1
	L-Ala-L-Ala-L-Ala	L-Ala-L-Ala-L-Ala-L-Ala	30.1
	L-Ala-L-Ala-L-Ala-L-Ala	L-Ala-L-Ala-L-Ala-L-Ala-L-Ala	22.8
	Gly-Gly	L-Ala-Gly-Gly	33.7
	Gly-Ala	L-Ala-Gly-L-Ala	35.1
	L-His-L-Ala	L-Ala-L-His-L-Ala	58.0
	L-Phe-Gly	L-Ala-L-Phe-Gly	34.0
	L-Leu-L-Ala	L-Ala-L-Leu-L-Ala	40.7
	L-Phe-L-Ala	L-Ala-L-Phe-L-Ala	24.8
L-Thr-OMe	Gly-Gly	L-Thr-Gly-Gly	8.4
Gly-OMe	L-Ala-L-Tyr	Gly-L-Ala-L-Tyr	0.6

(Example 30) Isolation of Gene for Peptide-forming Enzyme Derived from *Sphingobacterium* sp.

Hereinafter, isolation of gene for a peptide-forming enzyme is described. The microbe used was *Sphingobacterium* sp. strain FERM BP-8124 (Depositary institution: the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International

Patent Organism Depositary, Address of depositary institution: Chuo Dai-6,
1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit
date: July 22, 2002). For the isolation of gene, *Escherichia coli* DH5α was
used as a host, and pUC118 was used as a vector.

5   (1) Preparation of Microbial Cells

Sphingobacterium sp. strain FERM BP-8124 (Depositary institution:
the independent administrative corporation, National Institute of Advanced
Industrial Science and Technology, International Patent Organism Depositary,
Address of depositary institution: Chuo Dai-6, 1-1 Higashi 1-Chome,

10   Tsukuba-shi, Ibaraki-ken, Japan, International deposit date: July 22, 2002) was
cultivated for 24 hours at 25°C on CM2G agar medium (containing glucose 50
g/l, yeast extract 10 g/l, peptone 10 g/l, sodium chloride 5 g/l, and agar 20 g/l,
pH 7.0). One loopful cells of the resulting microbial cells was inoculated into a
500 ml Sakaguchi flask containing 50 ml of CM2G liquid medium (the

15   aforementioned medium excluding agar) followed by shaking cultivation at
25°C.

(2) Preparation of Chromosomal DNA from Microbial Cells

50 ml of culture broth was centrifuged (12,000 rpm, 4°C, 15 minutes) to
collect the microbial cells. A chromosomal DNA was then obtained from the

20   microbial cells using the Qiagen Genomic-Tip System (Qiagen) based on the
procedure described in the manual therefor.

(3) Preparation of Probe DNA Fragment by PCR

A DNA fragment containing a portion of gene for the peptide-forming
enzyme derived from *Empedobacter brevis* FERM BP-8113 (Depositary

25   institution: the independent administrative corporation, National Institute of

Advanced Industrial Science and Technology, International Patent Organism

Depositary, Address of depositary institution: Chuo Dai-6, 1-1 Higashi

1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date:

July 8, 2002) was obtained by the PCR method using LA-Taq (manufactured

5   by Takara Shuzo).   A PCR reaction was then carried out by using primers

having the base sequences of SEQ ID NOs: 3 and 4 to the chromosomal DNA

obtained from *Empedobacter brevis* strain FERM BP-8113 (Depositary

institution: the independent administrative corporation, National Institute of

Advanced Industrial Science and Technology, International Patent Organism

10   Depositary, Address of depositary institution: Chuo Dai-6, 1-1 Higashi

1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit transfer date:

July 8, 2002).

The PCR reaction was carried out by using Takara PCR Thermal

Cycler – PERSONAL (Takara Shuzo) for 30 cycles under the following

15   conditions.

94°C   30 seconds

52°C   1 minute

72°C   1 minute

After the reaction, 3 µl of reaction mixture was applied to 0.8% agarose

20   electrophoresis.   As a result, it was verified that a DNA fragment of about 1.5

kb was amplified.

(4) Cloning of Gene for Peptide-forming enzyme from Gene Library

In order to obtain the full-length gene for peptide-forming enzyme,

southern hybridization was carried out by using the DNA fragment amplified in

25   the aforementioned PCR procedure as a probe.   The procedure of southern

hybridization are explained in Molecular Cloning, 2nd edition, Cold Spring Harbor Press (1989).

The approximately 1.5 kb DNA fragment amplified by the aforementioned PCR procedure was separated by 0.8% agarose

5   electrophoresis.   The target band was then cut out and purified.   This DNA fragment was labeled with digoxinigen as probe by using DIG High Prime (manufactured by Boehringer-Mannheim) based on the procedure described in the manual therefor.

After allowing the chromosomal DNA of *Sphingobacterium* sp.

10   obtained in the step (2) of the present Example 30 to react with restriction enzyme *Sac*I at 37°C for 16 hours to completely digest the DNA, the resultant was electrophoresed on 0.8% agarose gel.   From the agarose gel after the electrophoresis, the electrophoresed chromosomal DNA was blotted onto a positively charged Nylon membrane filter (manufactured by Roche

15   Diagnostics), followed by treatments consisting of alkali denaturation, neutralization, and immobilization.   Hybridization was carried out by using EASY HYB (manufactured by Boehringer-Mannheim).   After pre-hybridizing the filter at 37°C for 1 hour, the digoxinigen-labeled probe prepared as described above was added and hybridization was carried out at 37°C for 16

20   hours.   Subsequently, the filter was washed twice at 60°C with 2 × SSC containing 0.1% SDS.

Detection of bands that hybridized with the probe was carried out by using the DIG Nucleotide Detection Kit (Boehringer-Mannheim) based on the procedure described in the manual therefor.   As a result, an about 3 kb band

25   was successfully detected that hybridized with the probe.

5 μg of the chromosomal DNA prepared in the step (2) of the present

Example 30 was completely digested with SacI. About 3 kb of a DNA was

separated by 0.8% agarose gel electrophoresis, the DNA was purified using

the Gene Clean II Kit (manufactured by Funakoshi), and dissolved in 10 μl of

5   TE.   4 μl of the resulting solution was mixed with pUC118 that had been

allowed to react treated with SacI at 37°C for 16 hours to completely digest it

and then treated with alkaline phosphatase (E. coli C75) at 37°C for 30 minutes

and at 50°C for 30 minutes and a ligation reaction was carried out by using the

DNA Ligation Kit Ver. 2 (manufactured by Takara Shuzo).   5 μl of this ligation

10   reaction mixture and 100 μl of competent cells of Escherichia coli DH5a

(manufactured by Takara Shuzo) were mixed to transform the Escherichia coli.

This was then applied to a suitable solid medium to produce a chromosomal

DNA library.

To obtain full-length gene for peptide-forming enzyme, the

15   chromosomal DNA library was screened by colony hybridization using the

aforementioned probe.   The procedure for colony hybridization is explained in

Molecular Cloning, 2nd edition, Cold Spring Harbor Press (1989).

The colonies of the chromosomal DNA library were transferred on a

Nylon membrane filter (Nylon Membrane for Colony and Plaque Hybridization,

20   manufactured by Roche Diagnostics), followed by treatments of alkali

denaturation, neutralization, and immobilization.   Hybridization was carried

out by using EASY HYB (manufactured by Boehringer-Mannheim).   After

pre-hybridizing the filter at 37°C for 1 hour, the aforementioned

digoxinigen-labeled probe was added, followed by hybridization at 37°C for 16

25   hours.   Subsequently, the filter was washed twice at 60°C with 1 × SSC

containing 0.1% SDS.

Detection of colonies hybridizing with the labeled probe was carried out by using the DIG Nucleotide Detection Kit (manufactured by Boehringer-Mannheim) based on the explanation described in the manual

5   therefor.   As a result, six strains of colonies were verified to have hybridized with the labeled probe.

(5) Base Sequence of Gene for Peptide-forming Enzyme Derived from *Sphingobacterium* sp.

Plasmids possessed by *Escherichia coli* DH5a were prepared from the

10   six strains of microbial cells that were verified to have hybridized with the labeled probe by using the Wizard Plus Minipreps DNA Purification System (manufactured by Promega) to determine the base sequence of a portion where hybridization with the probe occurred and nearby was determined.   The sequencing reaction was carried out by using the CEQ DTCS-Quick Start Kit

15   (manufactured by Beckman-Coulter) based on the procedure described in the manual therefor.   In addition, electrophoresis was carried out by using the CEQ 2000-XL (manufactured by Beckman-Coulter).

As a result, it revealed that an open reading frame that encodes peptide-forming enzyme did exist.   The full-length base sequence of gene for

20   the peptide-forming enzyme derived from *Sphingobacterium* sp. along with the corresponding amino acid sequence is shown in SEQ ID NO: 11. Peptide-forming enzyme derived from *Sphingobacterium* sp. exhibited a homology of 63.5% at the amino acid sequence level to the peptide-forming enzyme derived from *Empedobacter brevis* (as determined using the BLASTP

25   program).

(Example 31) Expression of Gene for Peptide-forming Enzyme Derived from *Sphingobacterium* sp. in *Escherichia coli*

The target gene was amplified by PCR using the chromosomal DNA of

5 *Sphingobacterium* sp. FERM BP-8124 (Depositary institution: the independent administrative corporation, National Institute of Advanced Industrial Science and Technology, International Patent Organism Depositary, Address of depositary institution: Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, Japan, International deposit date: July 22, 2002) as a

10 template and the oligonucleotides shown in SEQ ID NOs: 13 and 14 as primers. This DNA fragment was treated with NdeI/XbaI, and the resulting DNA fragment and an NdeI/XbaI treatment product of pTrpT were ligated. *Escherichia coli* JM109 was then transformed with this ligation solution, and strains having the target plasmid were selected from ampicillin-resistant strains.

15 The plasmid was designated as pTrpT_Sm_aet.

One loopful cells of *Escherichia coli* JM109 having pTrpT_Sm_aet was inoculated into an ordinary test tube containing 3 ml of a medium (glucose 2 g/l, yeast extract 10 g/l, casamino acid 10 g/l, ammonium sulfate 5 g/l, potassium dihydrogen phosphate 3 g/l, dipotassium hydrogen phosphate 1 g/l,

20 magnesium sulfate heptahydrate 0.5 g/l and ampicillin 100 mg/l) and the cultivation was performed at 25°C for 20 hours. It was verified that a cloned gene having an L-alanyl-L-glutamine production activity of 2.1 U per ml of culture broth was expressed by *Escherichia coli*. Furthermore, no activity was detected for a transformant containing only pTrpT used as a control.

25 (Prediction of Signal Sequence)

When the amino acid sequence of SEQ ID NO: 12 described in the Sequence Listing was analyzed with the Signal P v 1.1 program (Protein Engineering, Vol. 12, No. 1, pp. 3-9, 1999), it was predicted that numbers 1 to 20 in amino acid sequence was operated as a signal to secrete into the

5　periplasm, while the mature protein was estimated to be downstream of amino acid number 21.

(Confirmation of Signal Sequence)

One loopful cells of *Escherichia coli* JM109, having pTrpT_Sm_aet, was inoculated into ordinary test tubes containing 50 ml of a medium (glucose

10　2 g/l, yeast extract 10 g/l, casamino acid 10 g/l, ammonium sulfate 5 g/l, potassium dihydrogen phosphate 3 g/l, dipotassium hydrogen phosphate 1 g/l, magnesium sulfate heptahydrate 0.5 g/l and ampicillin 100 mg/l) and main cultivation was performed at 25°C for 20 hours.

Hereinafter, procedures after centrifugal separation were carried out

15　either on ice or at 4°C.　After the cultivation, the microbial cells were separated from the culture broth by centrifugation, washed with 100 mM phosphate buffer (pH 7), and then suspended in the same buffer.　The microbial cells were then subjected to ultrasonic disrupting treatment for 20 minutes at 195 W, the ultrasonically disrupted solution was centrifuged (12,000

20　rpm, 30 minutes) to remove the debris and obtain a soluble fraction.　The resulting soluble fraction was applied to a CHT-II column manufactured by Biorad) pre-equilibrated with 100 mM phosphate buffer (pH 7), and enzyme was eluted at a linear concentration gradient with 500 mM phosphate buffer. A solution obtained by mixing the active fraction with 5 fold volumes of 2 M

25　ammonium sulfate and 100 mM phosphate buffer was applied to a

Resource-PHE column (manufactured by Amersham) pre-equilibrated with 2 M ammonium sulfate and 100 mM phosphate buffer, and an enzyme was eluted at a linear concentration gradient by 2 to 0 M ammonium sulfate to obtain an active fraction solution.   As a result of these procedures, it was verified that

5   the peptide-forming enzyme was electrophoretically uniformly purified.

When the amino acid sequence of the aforementioned peptide-forming enzyme was determined by Edman's decomposition method, the amino acid sequence of SEQ ID NO: 15 was obtained, and the mature protein was verified to be downstream of amino acid number 21 as was predicted by the SignalP v

10   1.1 program.

INDUSTRIAL APPLICABILITY

According to the present invention, tripeptides can be produced easily using enzymes.   According to the method of the present invention, peptides

15   that are equal to or longer than tripeptides can be produced easily, inexpensively in high yields while mitigating complex synthesis methods such as introduction and elimination of protecting groups.

Sequence Listing

20   SEQ ID NO: 3: Synthetic primer 1

SEQ ID NO: 4: Synthetic primer 2

SEQ ID NO: 5: Gene encoding a peptide-forming enzyme

SEQ ID NO: 7: Synthetic primer for preparing pTrpT

SEQ ID NO: 8: Synthetic primer for preparing pTrpT

25   SEQ ID NO: 9: Synthetic primer for preparing pTrpT_Gtg2

SEQ ID NO: 10: Synthetic primer for preparing pTrpT_Gtg2

SEQ ID NO: 11: Gene encoding a peptide-forming enzyme

SEQ ID NO: 13: Synthetic primer for preparing pTrpT_Sm_aet

SEQ ID NO: 14: Synthetic primer for preparing pTrpT_Sm_aet

5